IRON IN DRINKING WATER OF PRE- AND POST-WEANED DAIRY CALVES

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ABSTRACT

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Well water might contain greater concentrations of ferrous iron (Fe^{2+}) than the suggested minimal tolerable amount for dairy calves (0.3 mg Fe/L). Ferrous iron is more biologically available compared with ferric iron (Fe^{3+}) and might have biologically negative effects on serum iron status, water and dry matter intake, and growth of calves. Our objective was to evaluate whether or not pre- and post-weaned calves show preference to waters containing Fe^{2+}, characterize Fe status through serial blood collections, measure water and starter pellet intake, and monitor growth through a series of experiments. In experiments 1 and 2, a non-parametric ranking design was used to evaluate pre- and post-weaned calves' preference for six concentrations of Fe^{2+} drinking water (0, 2, 4, 8, 12, or 20 mg Fe/L). Water and dry matter intake were measured 3 times per day. There was slight kappa agreement ($\kappa = 0.03$) of water ranking among pre-weaned calves and moderate kappa agreement ($\kappa = 0.36$) of water ranking among post-weaned calves.. Pre-weaned calves ranked 0 mg Fe/L treatment 1^{st} or 2^{nd}. Post-weaned calves preferred water with 0 mg Fe/L compared with water with added Fe^{2+}. In experiment 3, serum Fe and total iron-binding saturation (TIBS) of pre-weaned calves increased with increasing Fe^{2+} treatment (0, 2, 4, 8, or 12 mg Fe/L). Drinking water and starter pellet intake increased by week, but was not affected by treatment. The Fe^{2+} treatments did not detrimentally affect pre-weaned calves between 28 and 56 d of age.

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KEY TO ABBREVIATIONS

ADG = average daily gain

BW = body weight

CBC = complete blood count

CHCM = cell hemoglobin concentration mean

DM = dry matter

DMI = dry matter intake

EPA = United States Environmental Protection Agency

Fe = iron

Fe^{2+} = ferrous iron

Fe^{3+} = ferric iron

Hb = hemoglobin

Hct = hematocrit

MCH = mean corpuscular hemoglobin

MCHC = mean corpuscular hemoglobin concentration

MCV = mean corpuscular volume

RDW = red blood cell distribution width

SE = standard error

SEM = standard error of the mean

TIBC = total iron-binding capacity

TIBS = total iron-binding saturation

TMR = totally mixed ration

UIBC = unsaturated iron binding capacity

INTRODUCTION

The effects of excessive dietary iron (**Fe**) intake on dairy calves are not well understood. Effects of Fe in drinking water are even less well known. Ferrous iron ($\mathbf{Fe^{2+}}$) is a soluble form that varies in concentration in ground water sources. Ferric iron ($\mathbf{Fe^{3+}}$) is an insoluble form common in feedstuffs. Ferrous iron is presumed to be more bioavailable than the ferric form although controlled research studies are lacking on this point. Typical dairy calf diets are formulated to only account for the dietary Fe^{+3} that is present and supplemented in starter pellets and milk replacer. Pre-weaned, large dairy breed (e.g., Holstein and Brown Swiss) calves consume between 2 and 4 L of free drinking water and 8 L of milk replacer per d; whereas, weaned calves might consume more than 12 L of free drinking water per d. If the Fe concentration of drinking water results in Fe intake in excess of requirement, it is unclear if this excess Fe could be detrimental to calves' health and growth. It also is unknown whether or not calves show aversion to drinking water that contains high concentrations of Fe. Chapter 1 of this thesis is a literature review addressing bioavailability, absorption and metabolism of Fe, and its effects on hematological factors in calves. The known effects of pharmacological doses of Fe provided in calf feeds and iron's effects on feed intake, water intake, and growth also are reviewed.

The Environmental Protection Agency (EPA, 2004) stated that 0.3 mg Fe/L was the maximum tolerable concentration for "good quality" drinking water. This limit is based on preference for human consumption and what is considered to be aesthetically pleasing. It was not set as a limit for any other animal species, including dairy cattle. It is not uncommon for well source drinking water to have Fe concentrations considerably greater than 0.3 mg/L, but it is unknown if calves show aversion to greater concentrations in water due to palatability or other reasons. Chapter 2 describes two experiments we conducted in which pre-weaned or post-

weaned calves were offered drinking water treatments containing six different concentrations of soluble Fe from ferrous lactate. Calves ranked their preferences based on water consumption.

The effects of daily Fe^{+2} intake at concentrations well above EPA (2004) recommendation on pre-weaned calves' growth and hematological variables are not well defined or understood. Chapter 3 describes an experiment conducted to evaluate the effects of five different experimental treatments (varying Fe concentrations from free drinking water and water used to prepare liquid milk replacer) on total Fe binding capacity, total serum Fe, complete blood count profile, starter dry matter intake, free drinking water consumption, and growth.

The information from this research is expected to provide better insight into how drinking water with varying Fe concentrations provided to pre- and post-weaned calves impacts preference and consumption of drinking water and feed, Fe status, and growth.

CHAPTER 1

LITERATURE REVIEW

INTRODUCTION

Little information is available regarding the effects of ferrous iron (Fe^{2+}) content of drinking water on calf drinking preference, growth, or health. Anecdotal evidence suggests that calf growth might be impaired when excessive Fe in farm water is consumed (D. K. Beede, personal communication). There are a couple of reasons why excessive Fe^{2+} in water might cause issues. There might be decreased water consumption due to smell or taste aversions of the drinking water or there might be an excessive amount of Fe absorbed from the drinking water. Either or both could lead to decreased water and feed intake, suboptimal growth, and negative impacts on health.

Studies have reported that calves show preference for feed types (Erickson et al., 2004; Miller-Cushon et al., 2014; Terré et al., 2016). Furthermore, calves have the ability to rank multiple kinds of feeds over a period of time (Erickson et al., 2004; Erickson et al., 2012). No research currently is available assessing calves' ability to discern drinking water with varying concentrations of quality factors (e.g., Fe) or how drinking water preference might influence water intake.

Iron transport proteins regulate Fe concentrations in the body and act as a defense mechanism to protect against oxidative stress caused by Fe. These proteins signal other protein and mineral transport cascades throughout the body. A study showed weaned calves (59 ± 3d of age) provided a ration containing 15-times greater Fe (as ferrous sulfate) than recommended by the NRC (2001) caused decreased intestinal barrier integrity, decreased average daily gain (ADG), decreased dry matter intake (DMI), and decreased feed efficiency (Hansen et al., 2010).

This suggests there is a maximum concentration at which dietary Fe could negatively impact calves less than 4 mo of age.

This review examines the importance of water quality, the effects of excessive dietary Fe intake in calves, and the Fe metabolism mechanisms in the pre-weaned and weaned calf.

WATER QUALITY FACTORS AS INFLUENCED BY IRON

Solubility of iron. Elemental Fe exists in two valence forms; ferric (**Fe^{3+}**) and ferrous (**Fe^{2+}**). Natural waters that are not exposed to oxygen (e.g., stagnant water or deep groundwater) create a reducing environment that results in stable Fe^{2+} as the primary oxidative state. As the water travels through well and pipe systems, Fe continues to interact with carbon molecules and form Fe^{2+}. The rate of Fe^{2+} formation is dependent on pH of water when pH is between 5 and 8; but, the rate of Fe^{2+} formation is independent of pH when pH is greater than 8 (Morgan and Lahav, 2007).

Ferrous-water is colorless and is highly reactive with oxygen (O_2). As water is exposed to O_2, Fe^{2+} is oxidized to Fe^{3+} and water turns a reddish color. Ferric iron is very insoluble. The solubility of the ferric Fe in water at physiological pH is 10^{-17} mol/L (Fontecave and Pierre, 1993). This creates Fe properties in water that are less aesthetically pleasing for human consumption (EPA, 2004).

Water aesthetic. Adverse effects of Fe in water are discoloration of water due to oxidation of Fe^{2+} to Fe^{3+}, Fe^{3+} sediment formation, and promotion of microbiological growth causing biofilm (Farkas et al., 2012). Texture, taste, and odor of the water change with these properties. A human sensory study found individual thresholds between 0.007 and 14.14 mg/L Fe^{2+} with an overall population threshold of 0.17 mg/L Fe^{2+} in water (Mirlohi et al., 2011).

The U.S. Environmental Protection Agency (EPA) determines water quality based on the concentration of inorganic elements and compounds, organic compounds, radionuclides, microorganisms, and chemicals detected via water analyses (EPA, 2004). Total dissolved solids (TDS) are a measurement of all combined organic and inorganic material that is suspended or settled in water. This includes Fe sediment. The EPA (2003) lists Fe as a secondary contaminant meaning it is not detrimental to human health at a maximum contaminant level (opposed to a primary drinking water contaminant), but its effects on water are unfavorable for use and consumption if the concentration of Fe exceeds the maximum contaminant level of 0.3 mg Fe/L. Increased water turbidity and discoloration can occur at 0.05 to 0.1 mg Fe/L (WHO, 1996).

Microorganisms that create a biofilm, possibly leading to damage of pipes and watering receptacles, also perpetuate poor water quality. *Siderophilic* bacteria in particular can give water a metallic taste and create sludge (Farkas et al., 2012). Combined with sediment formation and discoloration, these properties of Fe and Fe-associated bacteria can create a poor quality of water by altering texture, taste, and smell.

Water quality. Using human water quality preferences to help understand how cattle might respond to water quality is a good starting point. However, it should not be assumed that aesthetic preferences rank similarly among animals. Differences in water quality experiences through exposure, drinking behavior, and total water consumption are likely important considerations.

There is insufficient research regarding Fe in drinking water for calves or adult cattle and how contaminants might influence water consumption. Cattle can consume their free drinking water requirements containing 0.3 mg Fe/L (EPA standard for Fe for humans) and remain within their dietary Fe requirements (NRC, 2001). Increasing (with ferrous lactate) Fe concentration of water from 4 to 8 mg Fe/L resulted in a 15.8 L (25 %) reduction in water intake of mid-lactation

dairy cows (Genther and Beede, 2013). Behaviors that suggest aversive interaction with water and not consumption of water (i.e., splashing, sniffing, or pushing of water containers) were not significantly different with increasing Fe water concentrations (Genther and Beede, 2013).

Horvath (1985) conducted the only other known water quality consumption tests in ruminants. Acid mine drainage was compared at pH 4 to 8 against tap water, and in a separate experiment, distilled water consumption was compared with acid water, hydrogen sulfate water, and ferric sulfate water (75 and 145 mg Fe/L) consumption. The various treatments were provided to groups of sheep without choice. Water consumption per $kg^{0.73}$ BW did not vary between pH levels, although tap water consumption was 61 to 99 mL/BW $kg^{0.73}$ greater than that of acidified water. Distilled water consumption (192 mL/BW $kg^{0.73}$) was greater than that of the 145 mg Fe/L ferric sulfate treatment (116 mL/BW $kg^{0.73}$) and consumption was similar by sheep of the 75 mg Fe/L ferric sulfate treatment (195 mL/BW $kg^{0.73}$). These results suggest that Fe-salts might have greater influence on water consumption than pH and that there might be a noticeable differences resulting from different concentrations of Fe in water.

Soluble Fe source. Iron can be found in water as Fe^{2+} and Fe^{3+} and in a number of combinations as a salt. Whether or not these various forms of Fe negatively impact water consumption by cattle, and if so, at what concentrations they cause aversion has not been extensively studied.

Iron valence and source and their effects on water consumption were researched with mid-lactation dairy cows. Cows were provided paired choices to evaluate the effect of Fe valence (Fe^{2+} and Fe^{3+}) and Fe source (ferrous lactate, ferrous sulfate, and ferrous chloride) (Genther and Beede, 2013). There was no difference in water intake, drinking duration, or frequency with water Fe treatments when provided in paired combinations of 0 mg/L or 8 mg/L of ferric sulfate or 8 mg/L of ferrous sulfate water treatments. There was slight reduction in time of water

consumption with ferrous chloride treatment (8 mg/L) when provided in combination with Fe-free water or ferric chloride water (8 mg/L) (treatment by time interaction). There were no treatment differences in frequency of consumption or non-consumptive behaviors when paired combinations of 8 mg/L of the three Fe^{2+} sources were provided. Overall, cows drank more frequently 0 mg Fe/L water compared with Fe^{2+}-supplemented water (Genther and Beede, 2013). Horvath (1985) found a decrease in water consumption by sheep when ferric sulfate was compared with ferric chloride provided at 145 mg Fe/L.

These studies suggest that valence and (or) salt-form of Fe in water might influence consumptive behavior of ruminants; however, concentration of Fe in water appears to have a greater effect on water intake than valence or salt-form; especially when compared with less adulterated tap or distilled water.

Variation of iron in ground water. Naturally occurring Fe concentrations in ground water are highly variable. An extensive United States Geological Survey study (DeSimone et al., 2009) conducted between 1994 and 2004 showed 19% of 2,100 private wells tested had Fe concentrations above the EPA recommended 0.3 ppm Fe (EPA, 2004). Socha et al. (2001) analyzed over 3,600 water samples from livestock operations across the USA; of those, over 40% of the samples contained Fe concentrations greater than 0.3 mg Fe/L. Within Indiana, Ohio, and Michigan, Fe concentrations in water ranged from 0 to 34.5 mg/L. On average, water contained 1.08 mg/L; about 3.6-times the EPA (2003) "good quality" standard. Large difference in Fe concentrations among farms in the upper Midwest suggest reason to question potential biological effects of high Fe^{2+} consumption by dairy cattle, and by calves in particular.

IRON NUTRITION OF CALVES

Dietary Iron

Iron absorption. The efficiency of Fe absorption varies. Some salt forms are absorbed more quickly and are more biologically available than other salt forms of Fe. Ferrous salts in particular are more readily absorbed and incorporated into cells (Fritz et al., 1970; Van Ravenswaay et al., 2001). Some Fe^{3+} can be reduced to Fe^{2+} in the abomasum before it crosses the mucosal membrane of the gastrointestinal tract into the blood stream (NRC, 2001).

Unabsorbed Fe is excreted primarily in feces (Ammerman et al. 1965; NRC, 2001) although very small amounts can be excreted in the urine (Ammerman et al. 1965). Absorption of orally supplemented Fe is highly regulated by the gastrointestinal tract and the animal's requirement for Fe. The requirement for Fe depends upon age, health, and physiological status (Hansen et al. 2010; Spears, 2003). Cytochrome b in the duodenal enterocyte reduces Fe^{3+} in digesta to Fe^{2+}. Ferrous Fe^{2+} is transported across the enterocyte into the cytoplasm via protein carrier, divalent metal transporter 1. In the cytoplasm Fe^{2+} can either be stored bound to ferritin protein or further exported from the cell by ferroportin and bound to transferrin to be transported in the blood (Hansen et al., 2010).

Toxicity. Several minerals require the divalent metal transporter 1 for intracellular transport. Excessive Fe can bind competitively to this transporter and inhibit the absorption of other important mineral elements such as Mn, Cu, and Zn (Hansen, 2010), although the exact mechanism of these metal interactions with the divalent metal transporter 1 in ruminants is unknown. Excessive Fe in the body also can cause oxidative stress of tissues (Hansen et al. 2010). Oxidative stress occurs when too many free radicals are formed and there are not enough anti-oxidants to neutralize them. This can lead to cellular death and eventually major organ dysfunction if too many cells are compromised.

Hansen et al. (2010) provided 8 wk-old weaned calves (73.8 ± 3.7 kg BW) with 0 or 2,300 mg Fe (750 mg Fe/ kg of dietary DM) using ferrous sulfate to assess the effects of excessive dietary Fe on Fe storage and intestinal permeability. At 56 d the calves were slaughtered. Excessive dietary Fe did not affect concentrations of Mn and Zn in the liver, duodenum, or heart. Iron content was greater in the liver of calves provided the high Fe diet. These calves also had a reduction in intestinal ferroportin protein, and an increase in duodenum permeability. The mechanism behind this is unknown; however, an increase in intestinal permeability can lead to dysregulated nutrient absorption and increase the calf's susceptibility to pathogens.

Highly regulated Fe absorption mechanisms are in place to safeguard against deficiency or toxicity. However, the evidence is inconclusive about what actually happens to dietary Fe in growing calves. The current upper limit of dietary Fe for young calves is 1,000 mg/kg of DM (NRC, 2001). From birth to 2 mo of age a calf can consume 0.5 to 2.0 kg of DM/d (NRC, 2001), making the toxic dose of Fe during this time between 500 and 2,000 mg of Fe/d.

For a calf to consume 500 to 2,000 mg of Fe/d would be difficult. This would be well above a calf's Fe requirement of 75 to 300 mg Fe/d (150 mg Fe/kg DM) during this time (NRC, 2001). Calf milk replacer and starter might contain up to 200 mg of Fe/kg to meet dietary requirements (anecdotal). A typical calf diet could contribute 160 mg Fe from milk replacer (0.8 kg powder) and 100 mg Fe from starter (0.5 kg/d DMI). A negligible amount of Fe typically would be contributed by water intake. However, Fe does accumulate in the body over time (Hansen et al., 2010), and Fe use and turnover rate in a calf are not well understood. The accumulation of Fe over time from consumption of drinking water and milk replacer made with well water containing Fe could interfere with normal biological functions.

Iron bioavailability. Not all Fe compounds are of equal biological availability when consumed (Fritz et al., 1970; Van Ravenswaay et al., 2001; NRC, 2001). This is an important consideration when evaluating the negative effects that excessive Fe consumption might have on calves. Potential issues include decreased feed intake and efficiency, poor growth, (Jenkins and Hidiroglou, 1987; Hansen et al., 2010) or signs of Fe toxicity (Jenkins and Hidiroglou, 1987).

Ferric iron is not efficiently absorbed from the gastrointestinal tract into the bloodstream and first must be converted to the more soluble Fe^{2+} form in the gastrointestinal tract (NRC, 2001). Thus, oxidation state of consumed Fe and any increase in Fe biological availability might be an important consideration. Few studies with ruminants have looked specifically at the bioavailability of different Fe sources or chemical salt-forms (Ammerman et al., 1967; Van Ravenswaay et al., 2001). There is one study that assessed the bioavailability of Fe sources provided to healthy or non-anemic calves (Ammerman et al., 1967).

Ammerman et al. (1967) compared the biological availabilities of Fe in ferric oxide, ferric chloride, ferrous carbonate, and ferrous sulfate in a series of studies. Nine dairy steer calves with an average age of 215 d and average BW of 110 kg were divided into a "Fe-depleted" group or a "non-Fe-depleted" group. Six calves considered "Fe-depleted" were fed a whole milk, low-Fe diet since birth and three steers considered to have normal Fe stores were provided a totally mixed ration (**TMR**) with hay from birth. Radioactive ferric chloride was orally administered in a capsule (73 mg Fe) to three Fe-depleted calves and three non-depleted calves, and ferric oxide was similarly administered to three or Fe-depleted calves. After 96 h all calves were slaughtered. Overall, greater amounts of Fe were found in the tissues of Fe-depleted calves; whereas, greater amounts of Fe were found in the feces of non-depleted calves. For the Fe-depleted calves, 54% of the radioactive Fe from ferric chloride was recovered in the feces; whereas, 14% of the Fe from ferric oxide was recovered in the feces. No urine samples contained

measurable activity from either radioactive Fe salt. There was tissue deposition of radioactive ferric chloride for all calves; however, no measurable radioactive ferric oxide was found in any of the calves' tissues.

In a subsequent study by Ammerman et al. (1967) six younger steer calves (172 d of age, 91 kg BW) were used after being fed a Fe-depletion diet similar to that in the previous study. Calves were orally dosed with 70 mg of radioactive Fe in ferric chloride or ferric oxide and slaughtered 168 h after dosing. At 96 and 168 h there were greater amounts of ferric chloride in the feces compared with ferric oxide. Rumen contents at 168 h contained 2% of the ferric chloride and 5.3% of the ferric oxide, suggesting that the ferric chloride might have been more easily absorbed into the bloodstream across the ruminal mucosa. Contrary to the previous study, measureable ferric oxide radioactivity was found tissues, although it was very low compared with ferric chloride concentrations. Urinary excretion of Fe was not recorded, but it is thought excreted through feces and urine.

In a third study, Ammerman et al. (1976) evaluated the absorption of ferrous sulfate, ferrous carbonate, ferric chloride, and ferric oxide on 24 wethers with an average BW of 39.2 kg. These sheep received nutritionally balanced rations and were not Fe-depleted. Radioactive Fe was orally dosed as a capsule containing 30 mg Fe of ferric chloride or ferric oxide, 70 mg Fe of ferrous sulfate, or 77 mg Fe of ferrous carbonate. Greater than 86% of Fe was recovered in the feces of wethers provided either ferric source. Less Fe from ferric oxide was excreted in urine compared with the Fe recovered in the urine from the other three Fe sources. Peak concentrations of Fe were found in serum between 6 and 24 h post-dosing. Peak concentrations were greatest for ferrous sulfate and ferric chloride, followed by ferrous carbonate, and then ferric oxide. It took between 24 and 48 h for radioactive Fe to appear in red blood cells. Uptake of Fe by red blood cells was similar for calves provided ferrous sulfate, ferrous carbonate, and ferric chloride.

These three sources had much greater Fe incorporation into red blood cells 96 h after dosing compared with ferric oxide. There also was less tissue deposition of Fe from ferric oxide compared with the other three sources.

Fritz et al. (1970) did a comprehensive study evaluating biological availability of various Fe^{3+} and Fe^{2+} sources provided to non-ruminants. Day old chicks and weanling rats with induced anemia were provided with one of 18 Fe^{2+} or Fe^{3+} salts. There was no association between animal species and the ability to better absorb one Fe salt over another. Relative biological values of Fe salts were determined by Fe's ability to return an anemic animals' hemoglobin and hematocrit values to within the normal range. Ferrous salts resulted in a greater relative biological value than Fe^{3+} salts. Of the Fe^{2+} salts, ferrous carbonate had the lowest relative biological value of 2, whereas ferrous sulfate and ferrous chloride had greater relative biological values of 100 and 98, respectively.

Van Ravenswaay et al. (2001) compared the bioavailability of ferrous sulfate and ferrous carbonate in 35 yearling wethers. The treatments were 0 mg/kg of dry matter (**DM**) supplementation from a Fe source, 300, 600, or 900 mg/kg of DM ferrous sulfate, or 600 mg/kg of DM ferrous carbonate mixed as part of total mixed rations (**TMR**). The supplement was provided for 30 d and then the wethers were euthanized to obtain liver, kidney, and spleen samples. Ferrous sulfate treatments had the greatest biological availability followed by the three carbonate sources.

These studies highlight important differences between sources of Fe^{2+} and Fe^{3+} and their bioavailability when provided to Fe-deficient or growing animals. Overall, Fe^{2+} sources consistently had greater biological availability with faster appearance of Fe in serum and red blood cells, and greater tissue deposition across species. These studies do not elucidate the

specific bioavailability of Fe^{2+} as part of water and how that might affect calf water consumption, health, or growth.

Dietary Fe recommendations. Some Fe^{3+} can be reduced to Fe^{2+} in the abomasum where Fe^{2+} can then chelate with a variety of compounds. Iron chelated with histidine, mucin, or fructose is more readily absorbed compared with Fe chelated to oxalate or phosphate. The concentration of Fe in the body dictates whether or not Fe is then bound to transferrin protein for transport to cells, or if Fe is bound to ferritin protein and eliminated from the body primarily in feces (NRC, 2001).

The rate at which Fe is absorbed and the amount of Fe that is absorbed into the bloodstream is determined by the animal's health and growth status (Ammerman et al. 1965; Spears, 2003; Hansen et al., 2010). If Fe^{2+} in water is more easily absorbed across the mucosa of the gastrointestinal tract, it could pose a greater toxicity threat than excessive Fe^{3+}, especially for young, growing calves.

The NRC (2001) indicates an absorption coefficient of 0.10 for Fe^{3+} and 0.40 for Fe^{2+} in adult ruminants. The absorption coefficient is dependent upon the form of Fe and total Fe intake. As calves consume more dry feed and transform from pre-ruminant to ruminant, the efficiency of Fe absorption decreases. This is thought to be a defense mechanism to protect ruminants against the potential large quantities of Fe found in forages and soil. Iron absorption efficiency also decreases in growing calves as the concentration of Fe in the diet increases, regardless of the biological requirement of Fe for growth has been met or not (NRC, 2001).

The estimated body store of Fe in a calf is between 18 to 34 mg/kg of BW (Bremner and Dalgarno, 1973). The estimated requirement of Fe for a 6-wk-old calf consuming 0.9 kg dietary DM/d and gaining 0.8 kg/d is 150 mg Fe/kg dietary DM (NRC, 2001).

Calves younger than 3 mo of age are undergoing extensive erythropoiesis. Dietary Fe influences this process (Egli and Blum, 1998; Brun-Hansen et al., 2006; Mohri et al., 2007). There is some research that evaluates the usefulness of supplemental Fe provided to growing calves (Mohri et al., 2004; Mohri et al., 2007). Some evidence suggests that dietary Fe provided above the recommended 150 to 180 mg Fe/kg dietary DM might increase red blood cell (RBC) count. It is unclear if the more efficiently absorbed form Fe^{2+} could be problematic.

Supplemental ferrous Fe. The effects of Fe supplementation on growth of weaned calves (Miller et al., 1991) and the effects of Fe on hematological variables in pre-weaned calves were evaluated (Jenkins and Hidiroglou, 1987; Mohri et al., 2004; Brun-Hansen et al., 2006). However, there is little research specifically evaluating potential adverse effects of Fe intake from water on feed intake and growth of pre-weaned calves.

An early study addressed the possible negative effects of excess Fe^{2+} in a liquid diet provided to growing calves (Jenkins and Hidiroglou, 1987). Feed consumption, BW, and apparent digestibility were evaluated using 40 Holstein and Ayrshire-Holstein bull calves provided milk replacer with supplemented Fe at greater than NRC (1980) recommendations. Calves were provided the treatments from 3 d of age to 6 wk of age, and Fe concentrations were varied using ferrous sulfate. Milk replacer treatments were 100, 500, 1,000, 2,000, or 5,000 mg Fe/kg DM, and average daily intakes resulted in consumption of 102, 485, 960, 1,980, or 4,250 mg of Fe/calf per day, respectively. Water was provided *ad libitum* and contained 1 mg/L of background Fe and contributed less than 1% of the daily Fe intake; no starter was provided. Milk replacer refusal was greatest for calves provided 5,000 mg/kg (6.3% refusal). Calves averaged 0.15 kg/d less average daily gain when provided 5,000 ppm of Fe compared with other treatments. However, weight gain was not linearly associated with Fe supplementation. Calves provided the 100 and 2,000 mg Fe/kg of DM/treatments gained 0.69 and 0.66 kg/d, respectively;

whereas, calves provided 500, 1000, and 5,000 mg Fe/kg of DM treatments only gained 0.26, 0.64, and 0.51 kg/d, respectively. Feed efficiency, apparent digestibility, and DMI were different between the 2,000 and 5,000 mg Fe/kg of DM treatments. Iron supplementation in milk replacer caused an increase in blood plasma Fe, although packed cell volume (**PCV**) was not different among treatments. The 2,000 and 5,000 mg Fe/kg DM treatments also caused an increase in Fe concentration of the liver. Iron toxicity was not thought to be a factor in this study. Effects on drinking water consumption were not measured.

Miller et al. (1991) evaluated the effects of ferrous carbonate on performance of Holstein heifer calves. From 1 to 9 wk of age they received 0, 500, 1,000, 2,000, or 4,000 mg Fe/kg of dietary DM as supplemental ferrous carbonate in a TMR. The control diet contained 170 mg Fe/kg DM from plant feedstuffs. Average Fe intakes over the 8-wk period were 293, 765, 1,670, 2,657, or 5,880 mg/calf per day, respectively for 0, 500, 1,000, 2,000, or 4,000 mg Fe/kg dietary DM of supplemental Fe from ferrous carbonate. Similar to Jenkins and Hidiroglou (1987), feed intake and rate of gain were not linearly related to Fe concentration. Feed intake was less when calves were provided the 2,000 mg Fe treatment and rates of gain when calves were provided 2,000 or 4,000 mg Fe/kg of dietary DM. Iron toxicity was not reported as an issue and calf performance, feed efficiency and health were not adversely affected. In the studies of Jenkins and Hidiroglou (1987) and Miller et al. (1991), calves consumed in excess of 2,000 mg of Fe^{2+} per day before feed intake decreased and subsequent average daily gain decreased. These Fe treatments are in great excess of what the majority of calves would consume in typical situations when fed a normal, balanced diet.

Iron analysis and oxidative stress

Serum Fe. Iron is an important constituent of hemoglobin, myoglobin, and enzymes. The regulation of bodily Fe during the first 3 mo of a calf's life is not well understood. However, numerous studies show that there is a great amount of erythropoiesis occurring during this time and that dietary Fe is necessary for normal processes of erythropoiesis (Egli and Blum, 1998; Mohri et al., 2007; Moozavian et al., 2010). Whether or not excessive dietary Fe could detrimentally affect these processes and negatively impact the health and growth of the calf is unknown. Holstein calves injected with 1,000 mg Fe as Fe-dextran at 2 d of age showed an increase in serum Fe by 28 d of age compared with a control group (Heidarpour et al., 2008). Whereas these studies are informative regarding the influence of Fe in young calves, they do not simulate what a continuous dietary intake of Fe above recommended concentrations (150 mg Fe/kg dietary DM; NRC 2001) would do to serum Fe concentration of a calf or what the limitation of serum Fe concentration is.

Total Fe-binding capacity. Total Fe-binding capacity (**TIBC**) is a way to assess serum Fe deficiency or overload in the blood. Iron binds transferrin protein to be transported in the blood. About one-third of the binding sites of transferrin are bound by Fe (De Jong et al., 1990). This Fe bound to transferrin is referred to as serum Fe (Moser et al., 1993). The amount of Fe that could potentially bind the unbound portions of transferrin is referred to as unsaturated Fe-binding capacity (**UIBC**). Together, UIBC and serum Fe are summed to compute TIBC. Serum Fe can be divided by TIBC and multiplied by 100 to determine total iron-binding saturation percentage (**TIBS**). If TIBC exceeds serum Fe, then there is no unbound Fe present in the serum and thus no assumed toxicity (Moser et al., 1993).

Ferritin is the protein that binds Fe for storage in the body. Serum ferritin can be measured in certain species to estimate total body stores of Fe. There is not an assay for bovine serum ferritin

at this time. Therefore, currently TIBC and percent saturation are the quantitative measurements used to estimate Fe stores in cattle.

Oxidative stress. Transferrin concentrations are influenced by several factors. Calves have greater concentrations of transferrin than cows and variable concentrations in cases of acute or chronic infectious disease (Moser et al., 1994). When transferrin is saturated or is not readily available in the body, Fe^{2+} remains unbound and creates reactive free radicals. These free radicles disrupt cellular membranes and allow penetration of Fe^{2+} to organ systems. This disruption allows Fe^{2+} to be reduced to Fe^{3+} and the hydrogen ions can further damage cells and alter DNA that can increase incidence of disease in animals (Albretson, 2006). Risk of free radicals formed by excessive intake of Fe can be assessed using TIBC and percentage of Fe saturation.

WATER NUTRITON OF CALVES

Water intake

Pre-weaned calves. Because milk or milk replacer is provided to dairy calves from birth to 1 to 2 mo of age, water consumption during the early pre-weaning phase is relatively small. However, water consumption is still an important component of a calf's total nutrient consumption and increases at a greater rate the second month of life and as more solid feed is consumed. *Ad libitum* water intake of Holstein calves reaches close to 2.5 L/d by 4 wk of age when consuming 3.78 L/d (0.432 kg DM) of milk replacer for the first 3 wk of age and 1.89 L/d (0.216 kg DM) of milk replacer at 4 wk of age (Kertz et al. 1984).

Water consumption is influenced greatly by the degree of mixing and dilution with milk replacer powder and frequency of milk replacer feeding. Finnish Ayrshire and Holstein bull calves provided a fixed amount of milk replacer liquid (7.5 L/d; 0.825 kg DM) and *ad libitum* drinking water (16 to 18^{o}C) from 3 to 10 wk of age consumed about 1 L/d by 4 wks of age, 2 L/d

by 8 wk of age, and then steadily increased total free drinking water intake to about 8 L/d by 75 d at weaning (Huuskonen et al., 2011). Calves provided colder drinking water (6 to 8^{o}C) consumed about 0.5 L/d by 4 wk of age, 1 L/d 8 wk of age, and 8 L/d by 75 d at weaning.

Quigley et al. (2006) evaluated various age-based milk replacer feeding programs for calves weaned at 28 or 42 d. Holstein bull calves (n = 120) were provided 3.8 L/d of milk replacer (0.454 kg DM) over two feedings until 28 d of age, or they were provided a volume of milk replacer based on age: 3.8 L/d (0.454 kg DM), 5.6 L/d (0.681 kg DM), 7.2 L/d (0.908 kg DM), and 3.8 L/d (0.454 kg DM) divided between two feedings and varied over four periods (0 to 7, 8 to 14, 15 to 31, and 32 to 41 d, respectively). Water was offered and *ad libitum* water intake was measured daily. Calf water intake was similar among treatments for the first 28 d with calves consuming up to 2 L/d by 4 wk of age. Calves that received milk replacer beyond 28 d of age steadily increased water consumption from 2 L/d at 28 d of age to 4 L/d at 35 d of age, and 8 L/d at 42 d age when they were weaned.

Contrary to Quigley et al. (2006), Huuskonen et al. (2011) did not observe water intake greater than 2 L/d by pre-weaned calves until 62 d of age. Although these calves were provided milk replacer via an automatic feeder and could have consumed up to 7.5 L of milk replacer per day until weaning at 75 d. This might have decreased the calves' need to drink free choice water.

These studies show that pre-weaned calves will consume free choice if given the chance, regardless of milk replacer quantity. It is still practice for many producers to withhold water for the first week or so of life with the understanding that calves are meeting their water requirements through milk replacer provision. While water requirements might be met through milk replacer, calves will drink 2 L of water/d during that time, which is much more than provided by milk replacer. Calves younger than 4 wk of age provided 3.8 L/d of milk replacer consumed 26 to 34% of their total water intake (milk replacer plus free choice water) through

free water consumption (Kertz et al. 1984; Quigley et al. 2006). Calves that remained on milk replacer beyond 28 d increased their free water consumption to 51% of total water intake by 35 d (Quigley et al. 2006). Even calves consuming up to 7.5 L of milk replacer per day consumed 20% of their total daily water as free choice drinking water (Huuskonen et al. 2011).

Weaned calves. Weaning has a significant influence on water consumption by calves and adequate water consumption is especially important at this time. Calves are no longer meeting part of their water requirements through milk replacer and can consume upwards of 10 L of water when they are 56 d of age (Quigley et al. 2006). Calves weaned at 28 d doubled their water intake from 2 to 4 L/d due to weaning (Quigley et al. 2006). A similar increase in water consumption from 1.5 to 2.5 L/d was found at 21 d of age when milk replacer was decreased from 3.8 L/d to 1.9 L/d, and again at 28 d when calves were weaned and water consumption increased to 3 L/d (Kertz et al. 1984). Calves weaned at 28 d of age continue to gradually increase their water intake to about 6 L at 35 d of age and 8 L/d by 42 d of age (Quigley et al. 2006). All calves in that study consumed 8 to 10 L/d between 42 and 56 d of age regardless of the pre-weaning milk replacer program and age at weaning.

Water requirements. Another important reason to encourage free water intake by calves is the strong relationship that exists between calf water consumption and starter intake (Kertz et al. 1984; Quigley et al. 2006). Calves provided *ad libitum* water had 3.6 kg greater starter consumption and 3.2 kg greater weight gain over the course of 4 wk compared with calves that were not provided water (Kertz et al. 1984). Quigley et al. (2006) found calves consumed 2 L of water per kg DM intake before d 28. Calves weaned at 28 d had an immediate increase in water intake to 4 L of water per kg DM intake compared with calves that remained on milk replacer; a ratio of 2 L/ 1 kg of DMI was maintained. Water intake to DM ratio continued to gradually increase over time for all calves. Calves weaned at 28 d had the ratio increased to 5 L of water

per kg of DM by d 35 and the other two groups of calves fed milk replacer increased to about 4 L of water per kg DM by d 35. When the latter group of calves was weaned at 42 d, their ratio increased to match the early-weaned calves (5 L of water per kg DM) and remained there until 56 d of age.

As free water intake greatly increases over the first 2 mo of life, it is important that adequate amounts of high quality water are provided at all times. The metabolic requirement of water for a healthy animal is 40 to 60 mL/kg BW per day (Wanamaker et al., 2008). Following this calculation, a calf 35 to 65 kg BW would require 2.1 to 3.9 L of water. This water can come from milk replacer or free drinking water assuming that the calf is in good health, provided a balanced diet, and is not heat-stressed. These other factors can change a calf's water requirement.

EARLY NUTRITION OF CALVES

Calf Growth

Nutritional requirements. Calves that grow faster will consume more starter and gain more BW. Heavier calves are less likely to leave the herd by first lactation and have greater milk production potential (Van De Stroet et al., 2016). The NRC (2001) indicates that target growth rate of a heifer should be 82% of her mature BW by the time she calves the first time. Daily energy and protein requirements of calves provided milk replacer and starter can vary depending upon daily gain goals. Maintenance metabolizable energy for calves is calculated as 0.100 Mcal/kg$^{0.75}$ body weight (**BW**; metabolizable energy). Pre-weaned calves averaging 60 kg BW by 28 d of age with a 0.2 kg/d gain require 2.8 Mcal and 102 g CP. Between 0.4 and 0.6 kg/d gain calves require 3.51 to 4.31 Mcal and 159 to 217 g CP, and at 0.8 kg/d gain calves require 5.16 Mcal and 275 g protein (NRC, 2001). Weaned calves averaging 90 kg with a 0.6 to 0.8 kg/d

gain require 6.07 to 7.19 Mcal, and 309 to 385 g CP, and weaned calves with a 0.9 kg/d gain require 7.78 Mcal and 423 g CP (NRC, 2001).

Water intake influence on starter intake and body weight gain. Several studies show the importance of maximizing pre-weaned calf nutrition to improve health of calves (Heinrichs and Heinrichs, 2011), increase growth rates (Zanton and Heinrichs, 2005; Cowles et al., 2006; Stamey et al., 2012), and increase subsequent milk production (Moallem et al., 2010; Soberon and Van Amburgh, 2013). Improvements in nutrition to maximize these areas of production are essential to maintaining healthy, good producing herds that can be profitable. This includes maximizing water and starter intake of calves.

Water intake and starter intake proportionally increase after 21 d of age (Kerts et al., 1984). Calves provided *ad libitum* water also consume more starter and gained more body weight compared with calves that were not provided *ad libitum* water (Kertz et al., 1984). An earlier study by Thickett et al. (1981) using 72 Freisian bull calves found a significant correlation between weight gain with water intake between 1 and 5 wk of age ($r = 0.056$, $P < 0.01$) and of starter intake with water intake between 0 and 5 wk of age ($r = 0.082$, $P < 0.01$). Additionally, for every increase in 1 L per day of water intake there was a 0.082 kg increase in daily starter intake and an increase of 0.056 kg of BW/d.

A study by Thomas et al. (2007) looked at ways to increase water intake of 21 to 28 d old calves by flavoring the water. Nine Holstein heifer claves in a 3 x 3 Latin Square design were provided *ad libitum* starter and water containing vanilla extract, orange extract, or no flavoring. The addition of orange or vanilla extract in water did not affect consumption of water compared with unflavored water, but there was an increase in starter intake by calves provided flavored water compared with calves provided unflavored water. Calves provided orange-flavored water

had the greatest starter intake (249 g/d) and the greatest BW gain (0.44 kg/d). There was no difference in drinking duration time, total drinking time, or time spent consuming starter of calves provided orange-flavored water compared with calves provided vanilla-flavored water. There was a trend ($P < 0.10$) for calves to spend less total time drinking water with vanilla flavoring but there was no difference in duration of drinking when compared with orange-flavored water.

Thomas et al. (2007) suggested that water factors other than total water consumption could influence starter intake. There might be a more adverse taste difference between vanilla-flavored water and starter compared with orange-flavored water and starter, which might have influenced greater starter intake by calves consuming orange-flavored water (Thomas et al., 2007). The same might be true for other water quality factors such as Fe concentration and how much particulate matter forms in the water from oxidation of Fe.

Milk replacer intake. Milk replacer allotments depend on the rate of gain and weaning ago goals. The minimum recommended milk replacer allotment is to provide 10% of the BW over two feedings per day (NRC, 2001). Calves provided *ad libitum* whole milk might consume nearly twice that amount. Jasper and Weary (2002) compared a restricted whole milk allotment (10% of body weight) to an *ad libitum* allotment with calves averaging 42 kg at 3 d of age. The calves provided milk replacer at 10% of their BW were provided 4.2 kg of whole milk at 3 d of age and increased to 5.7 kg of whole milk by 36 d of age. Calves allowed *ad libitum* whole milk intake consumed 9 kg by 7 d of age and continued to consume 9 to 10 kg of whole milk by 36 d of age. This study shows that calves have the ability to consume greater amounts of milk replacer beyond the recommended amount of 10% of BW per day. Whereas, the amount of milk replacer might affect starter intake, pre-weaned average daily gain, and BW at time of weaning (Jasper and Weary, 2002; Huuskonen et al., 2011), it is unclear if the amount of milk replacer

significantly affects the rate of gain of post-weaned calves (Jasper and Weary, 2002; Huuskonen et al., 2011). Using the NRC (2001) recommendation of 10% of BW per day of milk replacer, a 28 d old calf weighing 60 kg should receive at least 6 kg of milk replacer per day so as to not significantly reduce growth.

Starter intake of pre-weaned calves. How early pre-weaned calves consume measurable amounts of starter greatly depends on the milk replacer program. Kertz et al., (1984) provided calves with 1.9 L (0.216 kg milk replacer DM) of milk replacer twice daily for 3 wk and then dropped to 1.9 L of milk replacer at same DM content for 1 wk. For calves averaging 0.272 kg/d or greater of BW gain, average starter intake was less than 0.2 kg/d for the first 10 d of age, 0.6 kg/d by 21 d of age, and 1.4 kg by 28 d of age. These calves weighed 44 kg at the start of the experiment and were provided the recommended amount of fluid milk replacer of 10% of BW per day. Jasper and Weary (2002) showed that calves provided whole milk at an amount 10% of their BW or *ad libitum* milk replacer (9 to 10 kg/d) consumed under 0.5 kg/d between 0 and 28 d of age. Starter intake for both groups of calves did not exceed 1 kg/d until after weaning at 42 d of age. During the weaning process from 37 to 42 d of age all calves were provided whole milk diluted with 10% more water each day until 100% water was reached on d 42. During this time the calves provided a set amount of fluid (10% of BW) consumed more starter (0.88 kg/d) compared with calves provided diluted *ad libitum* whole milk (0.67 kg/d starter). Similarly, calves provided 2 L of whole milk twice per day consumed between 0.5 and 0.7 kg/d of starter between 28 d (58 kg BW) and 49 d of age (Thomas et al., 2007).

Milk replacer or whole milk typically provides the majority of the caloric needs (Mcal) to a calf. Encouraging starter consumption is a way to improve daily rate of gain and potentially wean calves sooner. On average, pre-weaned calves provided milk replacer at the minimum allotment of 10% of body weight per day might consume 0.2 to 0.5 kg of starter per day by 28 d

of age and this slowly increased to just less than 1 kg/d by a typical weaning age of 7 to 8 wk of age.

Body weight gain of pre-weaned calves. Average daily gain of pre-weaned calves is dependent on the plain of nutrition provided. Calves consuming more than 9 kg of whole milk per day had decreased starter intake, but increased average daily gain compared with calves provided up to 5.7 kg of whole milk per day (Jasper and Weary, 2002). Holstein heifer calves consuming 9 to 10 kg of whole milk *ad libitum* by 36 d of age had an average daily gain of 0.78 kg/d (Jasper and Weary 2002). Similarly, Ayrshire and Holstein bull calves provided 7.5 L of milk replacer (percentage of milk replacer no reported) gained an average of 0.7 kg/d between 20 and 75 d of age (Huuskonen et al., 2011). Holstein bull calves provided a varying amount of milk replacer 454 g of DM/d (3.8 L/d), 681 of DM g/d (5.6 L/d), 908 od DM g/d (7.2 L/d), and 454 g of DM/d (3.8 L/d) at 0 to 7, 8 to 14, 15 to 31, and 32 to 41 d of age, respectively, had similar average daily gains (Quigley et al., 2006). These calves averaged 0.468 kg of BW gain/d from 0 to 28 d of age and 0.728 kg of BW gain/d from 29 to 56 d of age. These studies show that there is a wide range of average daily gains for calves consuming an adequate plane of nutrition. An acceptable average daily gain for pre-weaned calves in their first month of age is 0.5 kg/d, whereas pre-weaned calves in their second month of age that are consuming more starter should have a greater average daily gain of about 0.7 kg/d.

Starter intake of weaned calves. While milk replacer allotment influences starter intake pre-weaning and during the weaning period, it does not appear to negatively impact starter or hay intake of calves once they are weaned as long as calves are provided at least 10% of their BW as fluid milk replacer per day (Jasper and Weary, 2002). Weaned calves that consumed between 9 and 10 kg of milk replacer per day consumed an average of 1.85 kg/d of starter (Jasper and

Weary, 2002). Calves weaned at 75 d of age averaged 2.6 kg/d of starter intake between 75 and 195 d of age (Huuskonen et al., 2011).

Body weight gain of weaned calves. It is inconclusive whether or not pre-weaning whole milk or milk replacer provisions significantly influence average daily gains of weaned calves (Jasper and Weary, 2002; Rosenberger et al., 2016). Calves consuming *ad libitum* whole milk (9 to 10 kg/d) weighed 81 kg at weaning and had an average daily gain of 0.85 kg/d after weaning at 43 d (Jasper and Weary, 2002). Holstein calves provided 8 and 10 L of whole milk/d weighed 65 to 70 kg at weaning (55 d) and had a weaned average daily gain of 1.23 and 1.32 kg/d, respectively (Rosenberger et al., 2016). The NRC (2001) indicates weaned calves weighing 80 kg should consume 1.51 to 2.18 kg/d DM to gain between 0.4 and 0.8 kg/d of weight, and that weaned calves weighing 90 kg should consume 2.09 to 2.68 kg/d DM to gain between 0.6 and 0.9 kg/d of weight.

Measurement landmarks. Several areas of the body can be measured to assess growth of a calf. Common areas of the body to measure are those that best predict the how well the calf can mature into a breeding animal; landmark measurements and values can be consistently identified from animal to animal (Koenen and Groen, 1998; Cerqueira et al., 2013). Measurements of growth to best track calves include BW, heart girth, wither height, hip height, hip width, and body length. Heart girth is measured with a tape immediately caudal to the forelegs and around the chest. Hip width is measured with a Vernier caliper measuring the distance between the left and right major trochanter. Body length is measured with a tape from dorsal scapular spine to the tuber ischii. Wither height is measured to the ridge between the scapula. Hip height is measured to the tuber coxae (Nugent et al., 1991; Cerqueira et al., 2013).

Growth rates. With animal genetics that vary significantly among farms, it is difficult to put a specific growth rate goal on body measurements such as wither height, hip height, hip width, and heart girth. These measurements are mostly used to associate with appropriate BW gain and approximate calving ease of maturing heifers greater than 6 mo of age (Kertz et al., 1998; Cerqueira et al., 2013). Body weight increases by 80% in the first 2 mo of life and then another 60% by 4 mo of age. At least 50% of mature wither height growth occurs within the first 6 mo of a calf's life (Kertz et al., 1998). Regression of BW on wither height, hearth girth, hip width, and body length have a $R^2 > 0.95$ with heart girth having the greatest relationship with BW indicating that multiple body measurements can be used to accurately assess growth (Heinrichs et al., 1992). Because specific measurements such as wither height and hip width vary greatly depending upon genetic background, there are not specific target goals for these growth variables in research. However, if these multiple measurements of growth are taken, they can be used to better confirm the accuracy of one another. This might be especially helpful when assessing calves of different conformations or having multiple people taking measurements.

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CHAPTER 2

EXPERIMENTS 1 AND 2: PREFERENCE OF PRE- AND POST-WEANED HOLSTEIN CALVES FOR FREE DRINKING WATER WITH VARYING CONCENTRATIONS OF IRON

ABSTRACT

Two water preference studies were conducted using six pre-weaned and six weaned Holstein calves provided waters containing varying concentrations of iron (**Fe**). In Exp. 1, individually penned pre-weaned calves aged 25 ± 2 d (mean ± range) were provided 8.3 L of milk replacer mixed at 13% DM, *ad libitum* pelleted starter, and six choices of non-treated waters *ad libitum* for a 3 d standardization period. The waters were then switched out for six choices of Fe-treated waters containing 0, 2, 4, 8, 12 or 20 mg Fe/L. Iron-water concentrations were formulated with ferrous lactate and made iso-lactate with lactic acid. The six choices of Fe-water treatments were provided *ad libitum* for 6 d and then the most consumed water was eliminated and replaced with an empty bucket. The most consumed water was determined by weight of waters remaining at the end of that period. The remaining five treatments were provided similarly for 5 d and then the most consumed eliminated at the end of that period. This was repeated until all Fe-water treatments had been eliminated over five periods (20 d). In Exp. 2, individually penned weaned Holstein calves aged 56 ± 3 d (mean ± range) were similarly studied, except the 7-d standardization period was conducted prior to introducing the six choices of Fe-water treatments to reduce chance of weaning stress effects. Fleiss's Kappa was used to determine inter-ranker agreement for each experiment. The overall κ for experiment 1 was 0.03 ($P < 0.001$) indicating slight agreement of ranking the six water treatments between six pre-weaned calves. There was fair agreement ($\kappa = 0.36$, $P < 0.001$) between pre-weaned calves

ranking 2nd and 4th place treatments of 0 mg Fe/L and 12 mg Fe/L, respectively. Pre-weaned calves averaged 0.03 L water intake/kg body weight by 48 ± 2 d of age (mean ± range). The overall κ for experiment 2 was 0.36 ($P < 0.0001$) indicating fair agreement between all six weaned calves and their rankings of the six water treatments. There was perfect κ agreement (κ = 1.00) in ranking 0 mg Fe/L water 1st and a fair κ agreement (κ = 0.36) in raking 12 mg Fe/L water 6th. Weaned calves averaged 0.12 L of water intake per kg of body weight by 83 ± 3 d of age (mean ± range).

INTRODUCTION

Iron (**Fe**) is an important mineral for cows; especially those that are still growing and require Fe for growth processes (Brun-Hansen et al., 2006; Mohri et al., 2007). Dairy calves are provided adequate concentrations of Fe in their diet through specially formulated milk replacers and starter pellets. A third source of Fe that is not typically considered a dietary source is ferrous iron (Fe^{2+}) that is naturally occurring in ground water used to make both the milk replacer suspension and provide free choice water.

The effects of excessive dietary Fe^{2+} intake from water consumption on dairy calves are unknown. Ferric iron (**Fe^{3+}**) is reduced in ground water to ferrous form (**Fe^{2+}**) that forms salts with other minerals. These Fe^{2+} salts are more readily absorbed and incorporated into cells of animals (Hanson et al., 2010) than Fe^{3+} forms and is presumed to be more bioavailable than Fe^{3+} forms (Fritz et al., 1970; Ravenswaay et al., 2001), although controlled research studies evaluating the effects of Fe^{2+} consumption from water sources are lacking on this point.

Pre-weaned calves consume between about 2 and 4 L of free drinking water (Kertz et al. 1984) and 8 L of milk replacer per d (Quigley et al., 2006); whereas, weaned calves might consume more than 10 L of free drinking water per d (Quigley et al., 2006). The Environmental Protection Agency (EPA, 2004) stated that 0.3 mg Fe/L was the maximum tolerable concentration for "good quality" drinking water. This limit is based on preference for human consumption and what is considered to be aesthetically pleasing. It was not set as a limit for any other animal species, including dairy cattle. It is not uncommon for well source drinking water to have iron concentrations considerably greater than 0.3 mg/L (Socha et al., 2003). This makes water a potentially significant source of dietary Fe^{2+}.

The Fe stores in the body are highly regulated (Hanson et al., 2010). Some Fe^{3+} is reduced to Fe^{2+} in the abomasum and Fe^{2+} is transported on transferrin protein to cells. If there are enough Fe stores in the body, then Fe is bound to ferritin protein and eliminated from the body primarily in feces (NRC, 2001). The efficacy and efficiency of Fe regulation and elimination in calves is not well understood. The estimated body store of Fe in a calf is between 18 to 34 mg/kg BW (Bremner and Dalgarno, 1973). The estimated requirements of Fe for a 6-wk-old calf consuming 0.9 kg dietary DM/d and gaining 0.8 kg/d requires 150 mg Fe/kg dietary DM (NRC, 2001).

If the Fe^{2+} concentration of drinking water intake is in excess of what a calf needs in its diet, it is unclear if this excess Fe^{2+} could be detrimental to the calves' health or if calves show aversion to drinking water that contains high concentrations of Fe^{2+}. It is generally accepted that plentiful volumes of clean water are necessary to promote adequate water intake, drive feed intake (Kertz, 1984), and thus promote a healthy start to a calf's life.

Previous studies show calves have the ability to discern feed sources and rank their choices of feed based on consumption (Erickson et al., 2004; Erickson et al., 2012). Genther and Beede, (2013) observed a reduction in water consumption of mid-lactation cows when water treated with ferrous lactate increased from 4 mg Fe/L to 8 mg Fe/L. The ability of calves to discern water quality choices has not been studied.

The objectives of these studies were to determine if pre-weaned and weaned calves could distinguish presumed "good quality" water from "poor quality" water that was altered with ferrous lactate to vary the Fe concentration of six water treatments from 0 mg Fe/L to 20 mg Fe/L. Our hypothesis was that water containing 0 mg Fe/L would be consumed the most by both pre-weaned and weaned calves and thus ranked higher than the waters containing greater

concentrations of ferrous lactate, and that waters containing 2 mg Fe/L or 4 mg Fe/L would be ranked higher than waters containing 8 mg Fe/L, 12 mg Fe/L, or 20 mg Fe/L.

MATERIALS AND METHODS

The Michigan State University (MSU) Institutional Animal Care and Use Committee approved all procedures for the experiments (approval no. 04/14-074-00). The experiments were conducted indoors at the MSU Metabolism Unit during June and July 2014. Calves were 28 ± 2 d or 63 ± 3 d (mean ± range) of age when entering the experimental period for experiments 1 or 2, respectively. All potential candidate calves were excluded from the experiments if health was compromised in any way at any time in life before the standardization period.

Calves were born at the MSU Dairy Teaching and Research Center (DTRC) and before the experiments began they were kept in individual outdoor calf hutches (PolyDome, Litchfield, MN or Calf-Tel Germantown, WI) and provided milk replacer (Cow's Match WarmFront® BOV BM DBZ, Land O'Lakes Animal Milk Products Co., Shoreview, MN) (the nutrient analysis is in Table 2.1), calf starter (Ampli-Calf, Purina Animal Nutrition, St. Louis, MO) (the nutrient analysis in Table 2.2), and drinking water from the DTRC well source (laboratory analysis is in Table 2.3). Six calves for experiment 1 were moved directly from the outdoor hutches to individual pens in the indoor Metabolism Unit for a 3-d standardization period before the 20-d experiment began. Subsequently (about 1 mo later) six different calves for experiment 2 were weaned (56 ± 3 d of age; mean ± range) and moved to an outdoor group pen for 7 d before entering a standardization period (7 d) in the indoor Metabolism Unit where they were kept in individual pens as described below.

Standardization periods

For experiment 1 four Holstein bull calves and two Holstein heifer calves fed milk replacer and calf starter were moved to the Metabolism Unit at 25 ± 2 d (mean ± range) of age for a standardization period of 3 d. Milk replacer was mixed as 13.5 % DM per manufacturer's

instructions using warmed (46 ^{o}C) well water from the DTRC. Calves were fed 2.4 L of mixed milk replacer suspension at 0800 and 1430 h, and 3.5 L at 2200 h. They were given 10 min to consume their allotment and then the bucket was removed and a drinking water bucket was put in place. In experiment 2, six post-weaned (weaned at 6 wk of age) Holstein heifer calves were moved indoors to the Metabolism Unit at 56 ± 3 d (mean ± range) of age for a standardization period of 7 d. Feeding and watering procedures during standardization were the same as for experiment 1 except no milk replacer was fed.

Pen arrangement for individual calves for experiments 1 and 2

In the Metabolism Unit calves were kept individually in pens (1.5 m x 2.6 m) on a concrete floor bedded with wood shavings 10 to 15 cm in depth. Three 4.7 L white plastic buckets were hung in metal bucket holders on each the front and back short sides (1.5 m in length) of the individual pens. All six drinking water buckets contained pre-weighed amounts of the same Metabolism Unit tap (well water) during the standardization period. Plastic Corex Drain Pipe (15 cm diameter) was fastened to the front edge of the water bucket holders to avoid spillage, defecation or urination into the drinking water buckets. A 4.7 L black plastic bucket containing starter feed (or milk replacer for a brief time at each feeding in experiment 1) hung in a metal bucket holder in the center of one long side (2.6 m in length) of each pen. The daily light: dark cycle was 16:8 h. The mean and maximum ambient temperatures in the Metabolism Unit were electronically maintained between 20.0 and 22.8^{o}C, respectively.

Experiment 1 and 2 offer of feed and water

Fresh pre-weighed house well water from the Metabolism Unit and calf starter were provided at each of three feedings (0800, 1430, and 2200 h) daily. Calves had access to starter

and water in all six white buckets at all times. This allowed them to adapt to having the freedom of choice of drinking from any of the six different buckets at any time. Sufficient house water from the Metabolism Unit was collected into 190 L plastic barrels at the start of each day to provide six calves in each experiment with common drinking water each day of the standardization period.

Treatments for experiments 1 and 2

All protocols and processes from standardization period remained the same for the experimental periods except that six different drinking water treatments were offered (one different treatment per bucket within each pen) with different Fe concentrations (0, 2, 4, 8, 12, or 20 mg/L) made using ferrous lactate hydrate (Sigma-Aldrich, Milwaukee, WI). All drinking water treatments were prepared using deionized and demineralized (DD) water using a commercial gel type, high capacity, high purity mixed ion exchange resin (Lewatit MN 91 resin, Besco Water Treatment, Inc., Mason, MI). Laboratory analysis of the DD water (0 mg/L Fe) is in Table 2.4). This water was used to prepare the other experimental treatments with added Fe. Treatments were made iso-lactate using 85% lactic acid (Avantor Performance Materials, Center Valley, PA). In the morning for each day of the experiment the iso-lactate ferrous Fe treatments were prepared as 500-mL stock solutions and added to 19 L buckets of DD water to make each of the treatments. The pH of iso-lactate Fe treatment solutions ranged from 5.26 (20 mg Fe/L) to 5.91 (0 mg Fe/L) pH. Tap water from the Metabolism Unit was used to make milk replacer suspension for experiment 1 (Table 2.3).

Experimental design

A non-parametric sequential elimination ranking design as described by Nombekela et al. (1994), Erickson et al. (2004), and Erickson et al. (2012) was used. Each experiment consisted of five periods over a total of 20 d. In day 1 of period 1, each calf was provided all six buckets of drinking water treatments with varying Fe concentrations completely randomized to spatial location in the six-bucket holders within each pen. Treatment waters and starter buckets were weighed back immediately before each of the three daily feeding times and water intake from each bucket was recorded. At 0800 h each successive day of a period water treatment buckets were re-randomized to spatial location within the pen of each calf. This process was repeated daily for 6 d of period 1. For each specific calf the drinking water treatment that was consumed in greatest amount over the entire experimental period was eliminated in the next experimental period. An empty white bucket replaced the treatment bucket that was eliminated in the previous period to maintain spatiality. The same procedures were followed for 5 d of period 2, 4 d of period 3, 3 d of period 4, and 2 d of period 5. Water treatments that had not been eliminated in previous experimental periods were offered *ad libitum* at all times. Two set of buckets were used and each day of the experiment water treatment buckets were scrubbed with soap and water and thoroughly rinsed with house water after each feeding and hung to dry before use the next feeding. For experiment 2 additional water treatments were made at the 2200 h feeding and watering time and used to replenish calves' treatment waters at 0300 h as needed.

Growth measurements

In each experiment body weight, length, heart girth, hip width, hip height, and wither height were measured 1 and 2 d prior to entering the first experimental period, and at d 13, 14, 19, and 20 of each experiment. Averages were taken of measurements made of the two

consecutive days. Body length was measured from scapula to tuber ischii, heart girth at the olecranon, and hip width from the points of the tuber coxae all with a measuring tape (Nugent et al., 1991). Wither height and hip height were measured with a cattle-measuring stick (Nasco, Fort Atkinson, WI). These data were not analyzed statistically, but used to monitor growth.

Water and feed sampling

During experimental data collection, a 100-mL water sample was collected at each feeding and watering (0800, 1430, and 2200 h) daily from each of the Fe water treatments and for the Metabolism Unit tap water used to make the milk replacer suspension (experiment 1 only). Individual water samples of the same treatment or source were pooled (100 mL) at the end of the period and acidified with 2 mL of nitric acid (EMD Millipore Corporation, Darmstadt, Germany). The water samples were analyzed for total recoverable Fe using the nitric acidified samples for each of the six ferrous Fe treatments, and Metabolism Unit tap water samples were analyzed for calcium carbonate ($CaCO_3$ for hardness), chloride (Cl), sulfate (SO_4), Ca, P, Mg, K, Na, Fe, Mn, Zn, and Cu (Cumberland Valley Analytical Services (Hagerstown, MD).

Milk replacer and starter sampled (about 100 g) each time a new bag was opened. Samples were composited every 2 wk and sent for nutrient analysis (Cumberland Valley Analytical Services, Hagerstown, MD). The nutrient analyses of milk replacer and starter are presented in Tables 2.1 and 2.2, respectively.

Statistical analysis

Daily consumption by each individual calf of each water treatment available during each successive experimental period was the primary measurement of interest. Each individual calf was the rater (ranker) in each experimental period and the subject is each water treatment. Fleiss'

kappa (κ) determined inter-rater agreement for consumption of the Fe-water treatments among the six calves in each experiment. The individual calves' consumption of each water treatment over each of the five periods was calculated and provided a number 1 through 6 ranking with 1 being the most consumed treatment for each calf and 6 being the least. This is shown in Tables 2.5 and 2.6 for experiments 1 and 2, respectively. These data were then transformed categorically. A "category 1" refers to the treatment consumed the most by a calf and thus was eliminated from that calf's pen at the end of period 1. The number of calves that ranked a treatment as a "category 1" was charted for frequency. This continued with "category 2" referring to the number of calves that preferred a particular treatment the next best. This frequency is determined similarly for six categories and is shown in Tables 2.7 and 2.8 for experiments 1 and 2, respectively.

Fleiss's κ is a modification of Cohen's κ (Fleiss, 1971) meeting the criteria that each drinking water treatment is rated by the same number of calves; however, not every calf must rank each water treatment. In each experiment, all six calves had the opportunity to rank the six water treatments; however, they did not have to differentiate a ranking among all of the treatments in the event of a tie. A procedural error in experiment 1 resulted in three calves not ranking their 5th and 6th choices of drinking water treatments. In experiment 2, all six calves ranked all six water treatments.

The equations to determine κ from the categorical data are those derived by Fleiss (1971). The mean of the proportion of raters that agree on the ranked position (p_a) of subject *i* with *m* as the number of subjects, *n* as the number of raters, and *k* as the number of categories $p_a = \frac{1}{mn(m-1)}\left[\sum_{i=1}^{n}\ \sum_{j=1}^{k}\ x_{ij}^2 - mn\right]$. The measure of error is defined as $p_\varepsilon = \sum_{j=1}^{k} q_j^2$ where $q_j = \frac{1}{nm}\sum_{i=1}^{n} x_{ij}$. Overall κ is defined as $\kappa = \frac{p_a - p_\varepsilon}{1-p_\varepsilon}$.

Simple average starter feed intake (Table 2.14) and body measurements (Table 2.15 and Table 2.16) are reported to illustrate that the calves were performing normally. However, they were not analyzed statistically because they were not the primary focus of these experiments and because of the experimental design used.

A drinking map was constructed to determine potential water consumption bias. Bias could occur based on where milk replacer or starter was placed, where doors to the building were located, or influence of neighboring calves in relation to placement of the waters in a pen. Each day the water that was consumed most was tallied in one of the six positions the water could have been placed in the individual pen. Since the most consumed water was removed each period and an empty bucket was used to replace that water and provide special similarity, the total number of times any of the waters could have been in one of six positions in the individual pens was tallied. The number of days a calf consumed the most water from a specific position was divided by the number of times a water was in that position to provide a percentage of times a calf consumed the most water each day from that specific placement.

RESULTS AND DISCUSSION

Experiment 1

The pre-weaned calves were 28 ± 2 d of age (mean ± range) and 72 ± 3.30 kg BW (mean ± SD) at the beginning of the 20-d experiment.

Water preference and intake variables. Inter-rater (among calves) agreement of ranking of Fe water treatments as determined by Fleiss' κ in poor agreement if $\kappa < 0$, slight agreement 0.01 to 0.20, fair agreement 0.21 to 0.40, moderate agreement 0.41 to 0.60, substantial agreement 0.61 to 0.80, and almost perfect agreement 0.81 to 1.00 (Fleiss, 1971). The overall κ for experiment 1 was 0.03 ($P < 0.001$; Table 2.9). Overall, pre-weaned calves in experiment 1 ranked Fe-water treatments with slight agreement.

The κ varied when Fe-water treatments were evaluated by category. The κ was 0.36 ($P < 0.001$) for categories 2 and 4, indicating pre-weaned calves were in "fair agreement" when ranking their 2nd and 4th place treatments. These treatments were 0 mg/L and 12 mg/L of Fe-water, respectively. Table 2.7 shows 4 calves ranked 0 mg Fe/L water in 2nd place, and 4 calves ranked 12 mg Fe/L in 4th place. The κ of categories 1, 3, 5, and 6 (the water treatments that were preferred 1st, 3rd, 5th, and 6th rankings) were in "poor agreement". Fourth and fifth place water rankings were missing for 8 mg/L for 3 calves, 20 mg/L for two the calves, and 4 mg/L for one calf. The frequency of calves agreeing on placement of these water treatments was less than or equal to 2 in these cases. Unfortunately these missing data contributed to an overall κ below "substantial agreement".

Average water intakes for each period for each calf in experiment 1 are shown in Table 2.10. Each calf determined which water treatments would be available to them in subsequent periods; therefore, which drinking water treatments were available after period 1 varied among

calves. It is unknown if the various combinations of Fe-water treatment available from day to day influenced daily water intake of each particular calf. Five of six calves' water consumption differed by less than 0.90 L (range of 3.1 to 4 L) with one calf consuming 1.8 L on average by the end of the 20-d experiment. Pre-weaned calves on experiment 1 averaged 0.03 L of water consumed per kg of body weight by the end of the 20-d experimental period. Calf 3 consistently consumed less water compared with the other calves and did not rank lower Fe concentration waters nor higher concentration Fe waters with consistency. All calves consumed 1.5 to 3 L of water per day by 35 d of age (the beginning of the experiment), and most calves increased water consumption over the course of 20 d. By about 50 d of age five of the calves were consuming more than 3 L of water. These daily water intake data are similar to those reported by Kertz et al. (1984) and Huuskonen et al. (2011) and suggest that water intake calves was within an acceptable range during the course of the experiment. A map of daily water intakes for each calf was evaluated by dividing the number of times a calf consumed the least or most water in a day from a particular spot by the number of times water was available in that spot over the 20-d experiment. The number of times an individual calf consumed the least or most water in a day from one particular spot varied greatly from 0 to 69%. However, there was no obvious pattern of consumption or aversion shown by all six of the calves that indicated there was a bias in spatial bucket placement within the pen in relation to water consumption.

Average daily starter intakes were similar among calves (Table 2.11). Average daily gain was 1.05 kg over the 21 d experiment. Calf starter intake and daily gain data are similar to those reported by Quigley et al. (2006) and Huuskonen et al. (2011) and suggest feed consumption and growth were within an acceptable range during the course of the experiment.

Experiment 2

Post-weaned calves were 63 ± 3 d (mean ± range) and 95 ± 9.73 kg BW (mean ± SD) at the beginning of the 20-d experiment.

Water preference and intake variables. The κ was 1.0 ($P < 0.001$) "almost perfect agreement" for category 1 and "fair agreement" for categories 3 (κ 0.28, $P < 0.01$), 5 (κ 0.28, $P < 0.01$), and 6 (κ 0.36, $P < 0.001$) in experiment 2. All calves ranked treatment (0 mg/L) as their first choice. Four of six of calves agreed on their 3rd (4 mg/L), 5th (8 mg/L), and 6th (12 mg/L) treatment choices. Across the entire experiment there was "fair agreement" in drinking water preference with κ ranking of "fair agreement" (Table 2.12).

Average water and calf starter intakes for experiment 2 are in Table 2.13 and Table 2.14, respectively. Water and feed intakes varied somewhat among calves, likely due to some difference in BW among calves. At the start of the experiment there was a 23 kg difference between the largest and smallest calf, and a 34 kg difference between the largest and smallest calf at the end of the experiment. On average calves consumed 0.12 L of water per kg of BW (SD ± 0.01) by 83 ± 3 d of age (mean ± range).

A map of daily water intakes for each calf was evaluated similar to experiment 1 over the 20-d experiment. The number of times an individual calf consumed the least or most water in a day from one particular spot varied greatly from 0 to 68%. However, there was no obvious pattern of consumption or aversion shown by all six of the calves that indicated there was a bias in spatial bucket placement within the pen in relation to water consumption.

Average daily gain for weaned calves was 1.56 ± 0.36 kg (average ± SD) over the 20-d experiment. Feed intake and average daily gain were typical for this age and breed of weaned calves (NRC, 2001) and above that for targeted BW of calves this age (Kertz et al. 1998).

Whereas, the overall κ was not greatest possible in experiment 2, having all calves rank water without added Fe (0 mg Fe/L) most preferred initially is notable. Furthermore, all calves ranked either 12 mg Fe/L or 20 mg Fe/L treatment in 6th place (last place). This suggests that the calves could detect differences in Fe-water treatment at least at the extremes. These weaned calves consumed more than 8 L of water in the first period and greater than 11 L of water by the last period of the experiment (Table 2.13). This is a much greater consumption of water than the pre-weaned calves in experiment 1. Because of greater overall water consumption by calves in experiment 2 they might have had greater opportunity to differentiate quantitatively among the Fe-water treatments compared with calves in experiment 1.

The overall κ is determined by a calculation that evaluates difference in an observed agreement by the calves (how each calf ranked each water treatment) and an agreement of water ranking that could be due to chance alone. In each experiment there was a possibility for six data points for each of six calves (36 data points total). Of the six missing data points due to procedural error in experiment 1, 3 were for 8 mg Fe/L water treatment resulting in the "poor agreement" for 5th and 6th rankings and greatly contributing to the overall κ below "substantial agreement" of the experiment. These data points were missing from the treatments that would likely be considered the calves' least two favorite water treatments. Calves were still able to provide rankings for Fe-water treatments for 0, 2, 4 and 8 mg Fe/L.

A similar study could be used to further evaluate calf water preferences in the future; although methods of water measurement should be improved. Difficulties in accurate water consumption measurement arose when calves would bump into the buckets or contaminate the waters with feces and urine. A more enclosed bunker for multiple water treatments could decrease spills and contamination. Randomizing the treatments within the pen each day ideally decreased the amount of error that could be attributed to spills and contamination by preventing

the same treatment from being in the same area that is more prone to these errors. It would be of interest to re-evaluate more pre-weaned calves so that all data could be attained and possibly a more substantial κ. While there is no repeatability evaluation for κ *per se* it would be of merit to evaluate more groups of calves in this type of a ranking design to determine a range of κ in this kind of experimental design. This would better elucidate the validity of preference findings of calves.

CONCLUSION

In conclusion, there was not substantial kappa agreement for ranking Fe water choices in both experiments with pre-weaned and post- weaned calves; although all of the post-weaned calves clearly preferred the water without Fe compared with the five Fe-water treatments. Even though experiment 1 was missing data for the 5^{th} and 6^{th} rankings, the first four rankings for all of the calves were still poor or fair kappa agreement and suggest that pre-weaned calves do not have a predilection towards water with or without ferrous Fe. Weaned calves had a perfect agreement ($\kappa = 1.00$) in ranking 0 mg Fe/L water 1^{st}, and a fair agreement ($\kappa = 0.36$) in ranking 12 mg Fe/L water last, suggesting a preference for water with lower concentrations of Fe when also provided water with a much greater Fe concentration.

APPENDIX A

TABLES REFERENCED IN CHAPTER 2

TABLES

Table 2.1. Laboratory analysis of milk replacer used in preliminary periods and experiment 1[1, 2]

Component	Percent
Moisture	7.5
DM	92.5
	Percent of DM
CP	27.1
ADF	0.5
Ash	11.03
Ca	0.81
P	0.76
Mg	0.13
K	2.1
Na	0.96
	mg/kg of DM
Fe	104
Mn	37
Zn	37
Cu	51
[1] Average of composite samples taken each week for 3 wk.	
[2] Cow's Match WarmFront® BOV BM DBZ, Land O'Lakes Animal Milk Products Co., Shoreview, MN.	

Table 2.2. Laboratory analysis of calf starter used in experiments 1 and 2[1, 2]

Component	Percent
Moisture	12.35
DM	87.65
	Percent of DM
CP	22.3
ADF	14.7
Ash	8.15
Ca	0.94
P	0.62
Mg	0.29
K	1.36
Na	0.50
	mg/kg of DM
Fe	209

Table 2.2 (cont'd)

Mn	142
Zn	226
Cu	48
[1] Average of composite samples taken each week for 3 wk.	
[2]Ampli-Calf, Purina Animal Nutrition, St. Louis, MO.	

Table 2.3. Laboratory analysis of house well water used to water calves before experiments 1 and 2[1]

Component	**mg/L**
Hardness, ppm $CaCO_3$	424
TDS[2]	682
Cl	165
SO_4	87.4
Ca	119.5
P	< 0.10
Mg	35.2
K	21.6
Na	8.58
Fe	2.11
Mn	< 0.05
Zn	0.05
Cu	< 0.01
[1] Average of composite samples taken at the beginning of each experiment.	
[2]TDS = total dissolved solids.	

Table 2.4. Laboratory analysis of deionized and demineralized water used to prepare drinking water treatments

Component	**mg/L**
Hardness, ppm $CaCO_3$	5.74
TDS[2]	< 0.5
Cl	< 2.2
SO_4	< 1
Ca	4
P	10
Mg	< 1
K	< 1
Na	< 0.1
Fe	< 1

Table 2.4 (cont'd)

Mn	< 1
Zn	< 0.01
Cu	< 0.01
[1] Average of composite samples taken at the beginning of each experiment.	
[2]TDS = total dissolved solids.	

Table 2.5. Experiment 1. Sequential ranking of six Fe-water treatments by six pre-weaned calves

Water Treatment (mg/L)	**Calf 1**	**Calf 2**	**Calf 3**	**Calf 4**	**Calf 5**	**Calf 6**
0	2	2	2	3	1	2
2	3	1	6	2	2	1
4	5	3	4	..	4	3
8	6	..[1]	1	..	5	..
12	4	4	5	4	3	4
20	1	..	3	1	6	..
[1] 5th and 6th water rankings for periods 4 and 5 not available for 3 calves.						

Table 2.6. Experiment 2. Sequential ranking of six Fe-water treatments by six post-weaned calves

Water Treatment (mg/L)	**Calf 1**	**Calf 2**	**Calf 3**	**Calf 4**	**Calf 5**	**Calf 6**
0	1	1	1	1	1	1
2	2	4	5	2	2	3
4	3	3	2	3	3	2
8	5	5	4	4	5	5
12	6	6	3	5	6	6
20	4	2	6	6	4	4

Table 2.7. Experiment 1. Categorical frequency transformation of six Fe-water treatments ranked by six pre-weaned calves

	Frequency					
Water Treatment (mg/L)	**Category 1[1]**	**Category 2**	**Category 3**	**Category 4**	**Category 5**	**Category 6**
0	1	4	1	0	0	0
2	2	2	1	0	0	1
4	0	0	2	2	1	0
8	1	0	0	0	1	1
12	0	0	1	4	1	0
20	2	0	1	0	0	1
Total	6	6	6	6	3	3

[1]Category 1 refers to the overall agreement or disagreement of the most consumed water treatment by all calves by the end of period 1. Categories 2 through 6 are for that of periods 2 through 6.

Table 2.8. Experiment 2. Categorical frequency transformation of six Fe-water treatments ranked by six post-weaned calves

	Frequency					
Water Treatment (mg/L)	**Category 1[1]**	**Category 2**	**Category 3**	**Category 4**	**Category 5**	**Category 6**
0	6	0	0	0	0	0
2	0	3	1	1	1	0
4	0	2	4	0	0	0
8	0	0	0	2	4	0
12	0	0	1	0	1	4
20	0	1	0	3	0	2
Total	6	6	6	6	6	6

[1]Category 1 refers to the overall agreement or disagreement of the most consumed water treatment by all calves by the end of period 1. Categories 2 through 6 are for that of periods 2 through 6.

Table 2.9. Experiment 1. Fleiss' Kappa (κ) statistical analysis of sequential ranking of six Fe-water treatments by six pre-weaned calves

	Category						
	1[1]	**2**	**3**	**4**	**5**	**6**	**Overall**[7]
Kappa(κ)[2]	-0.04	0.36	-0.12	0.36	-0.09	-0.09	0.03
SE[3]	0.11	0.11	0.11	0.11	0.11	0.11	0.01
z-statistic[4]	-0.38	3.42	-1.14	3.42	-0.86	-0.86	3.69
***P*-value**[5]	0.70	< 0.001	0.25	< 0.001	0.39	0.39	< 0.001
Lower CI[6]	-0.25	0.15	-0.33	0.15	-0.30	-0.30	0.02
Upper CI	0.17	0.57	0.09	0.57	0.12	0.12	0.05

[1]Category 1 refers to the overall agreement or disagreement of the most consumed water treatment by all calves by the end of period 1. Categories 2 through 6 are for that of periods 2 through 6.
[2]Degree of inter-rater agreement. Poor agreement if < 0, slight agreement 0.01 to 0.20, fair agreement 0.21 to 0.40, moderate agreement 0.41 to 0.60, substantial agreement 0.61 to 0.80, and almost perfect agreement 0.81 to 1.00 (Fleiss, 1971).
[3]Standard error of the mean.
[4]Categorical Kappa (κ) divided by SE.
[5]Determined by z-statistic.
[6]Lower and upper confidence interval.
[7]Statistical analysis across all categories.

Table 2.10. Experiment 1. Average daily water intake (L) of six pre-weaned calves by period

Days (period)	**Calf 1**	**Calf 2**	**Calf 3**	**Calf 4**	**Calf 5**	**Calf 6**	**± SD**
1-6 (1)	2.8	1.4	1.5	2.7	3.1	2.0	0.7
7-11 (2)	3.8	2.5	1.6	2.7	5.4	3.1	1.3
12-15 (3)	3.3	2.7	2.1	3.4	4.4	3.8	0.8
16-18 (4)	5.1	3.1	2.2	2.3	4.1	3.3	1.1
19-20 (5)	3.2	3.7	1.8	3.1	3.2	4.0	0.8

Table 2.11 Experiment 1. Average daily starter feed intake (kg) of six pre-weaned calves by period

Days (period)	Calf 1	Calf 2	Calf 3	Calf 4	Calf 5	Calf 6	± SD
1-6 (1)	0.1	0.4	0.4	0.6	0.59	0.64	0.2
7-11 (2)	0.6	0.7	0.8	0.9	1.07	0.71	0.2
12-15 (3)	0.7	0.8	0.8	1.3	1.07	1.04	0.2
16-18 (4)	1.1	0.9	1.0	0.9	1.20	1.03	0.1
19-20 (5)	1.0	1.2	1.2	1.0	1.22	1.24	0.1

Table 2.12. Experiment 2. Fleiss' Kappa (κ) statistical analysis of sequential ranking of six Fe-water treatments by six post-weaned calves

	Category						
	1[1]	**2**	**3**	**4**	**5**	**6**	**Overall[7]**
Kappa (κ)[2]	1	0.12	0.28	0.12	0.28	0.36	0.36
SEM[3]	0.11	0. 11	0. 11	0. 11	0. 11	0. 11	0.05
z-statistic[4]	9.49	1.14	2.67	1.14	2.67	3.42	7.64
***P*-value[5]**	<0.001	0.25	0.01	0.25	0.01	< 0.001	<0.001
Lower CI[6]	0.79	-0.09	0.07	-0.09	0.07	0.15	0.27
Upper CI	1.21	0.33	0.49	0.33	0.49	0.57	0.4

[1]Category 1 refers to the overall agreement or disagreement of the most consumed water treatment by all calves by the end of period 1. Categories 2 through 6 are for that of periods 2 through 6.
[2]Degree of inter-rater agreement. Poor agreement if < 0, slight agreement 0.01 to 0.20, fair agreement 0.21 to 0.40, moderate agreement 0.41 to 0.60, substantial agreement 0.61 to 0.80, and almost perfect agreement 0.81 to 1.00 (Fleiss, 1971).
[3]Standard error of the mean.
[4]Categorical Kappa (κ) divided by SE.
[5]Determined by z-statistic.
[6]Lower and upper confidence interval.
[7]Statistical analysis across all categories.

Table 2.13. Experiment 2. Average daily water intake (L) of six post-weaned calves by period

Days (period)	Calf 1	Calf 2	Calf 3	Calf 4	Calf 5	Calf 6	± SD
1-6 (1)	13.7	10.1	13.5	10.2	12.3	8.8	2.0
7-11 (2)	18.1	9.6	16.6	12.5	13.9	10.7	3.3
12-15 (3)	16.5	8.9	15.6	14.2	14.2	11.6	2.8
16-18 (4)	17.8	9.3	18.8	13.3	12.7	12.3	3.6
19-20 (5)	19.0	11.6	14.6	15.0	14.4	12.6	2.6

Table 2.14. Experiment 2. Average daily feed intake (kg) of six post-weaned calves by period

Days (period)	Calf 1	Calf 2	Calf 3	Calf 4	Calf 5	Calf 6	± SD
1-6 (1)	3.8	2.7	4.2	3.2	3.5	3.0	0.6
7-11 (2)	4.5	2.3	4.3	3.7	3.8	2.9	0.9
12-15 (3)	4.5	3.1	4.6	4.0	4.3	3.4	0.6
16-18 (4)	5.0	3.2	5.6	4.1	3.9	3.5	0.9
19-20 (5)	5.5	3.5	5.3	4.2	4.4	3.8	0.8

Table 2.15. Experiment 1. Change in body measurements of pre-weaned calves 28 ± 2 d of age (mean ± range) from start to end of 20-d experimental period

Measurement[1]	Start of Experiment (average)	± SD		End of Experiment (average)	± SD
Weight (kg)	72.0	3.3		96.5	3.7
Hip Width (cm)[2]	24.7	0.7		248.9	4.9
Hip Height (cm)	91.2	1.6		12.3	0.5
Wither Height (cm)	88.0	0.8		94.2	1.6
Heart Girth (cm)	97.9	1.7		108.4	1.5
Length (cm)[3]	86.3	2.8		100.1	1.9

[1]Each measurement was taken 2 consecutive days and averaged at the start of the experiment (d 1-2) and at the end of the experiment (d 19-20).
[2]Hip width measured from the points of the tuber coxae.
[3]Body length was measured from scapula to tuber ischia.

Table 2.16. Experiment 2. Change in body measurements of post-weaned calves 63 ± 3 d of age (mean ± range) from start to end of 20-d experimental period

Measurement	Start of Experiment (average)	± SD		End of Experiment (average)	± SD
Weight (kg)	95.0	9.7		123.0	13.3
Hip Width (cm)	25.2	1.2		33.4	2.5
Hip Height (cm)	92.8	0.0		92.8	0.0
Wither Height (cm)	88.5	0.0		88.5	0.0
Heart Girth (cm)	103.6	3.0		115.7	3.4
Length (cm)	94.5	2.9		102.8	3.4

[1]Each measurement was taken 2 consecutive days and averaged at the start of the experiment (d 1-2) and at the end of the experiment (d 19-20).
[2]Hip width measured from the points of the tuber coxae.
[3]Body length was measured from scapula to tuber ischia.

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CHAPTER 3

EFFECTS OF FERROUS (Fe^{2+}) IRON IN DRINKING WATER AND MILK REPLACER ON IRON STATUS, INTAKE, AND GROWTH OF PRE-WEANED CALVES

ABSTRACT

The concentration of ferrous iron (**Fe^{2+}**) in well water can vary greatly and might exceed the recommended upper tolerable limit (0.3 mg Fe/L). The impacts of water containing Fe^{2+} greater than this limit in free drinking water and milk replacer for pre-weaned calves are unknown. The objective was to determine the effects of Fe^{2+} concentrations in free drinking water and milk replacer on iron status, water and feed intake, and growth of pre-weaned calves using Fe^{2+} concentrations in the range that have been reported in dairy farm well water samples. Twenty-five bull and 35 heifer calves were each provided 0, 2, 4, 8, or 12 mg Fe/L treatments in a completely randomized block design from 28 to 56 d of age. Serum Fe and total iron-binding saturation (**TIBS**) increased with increasing iron in drinking water and milk replace, and serum Fe, TIBS, and total iron-binding capacity (**TIBC**) increased by wk. Serum Fe remained within normal reference ranges and TIBS exceeded 50% for 8 and 12 mg Fe/L treatments, and transferrin was not completely saturated for any treatment. There was a quadratic effect of treatment on hemoglobin concentration that was especially pronounced by the end of the 4-wk experiment. Treatment did not affect drinking water intake or dry matter intake of starter and both increased over time. Calves provided 4 mg Fe/L had greater BW than other treatments. Average daily gain (**ADG**) and heart girth were greater for bull calves compared with heifer calves. This experiment suggests that increasing concentrations of Fe^{2+} in drinking water and milk replacer can increase iron status indicators without detriment to the calf and affect BW without impacting drinking water, dry matter intake, and growth.

INTRODUCTION

Iron (Fe) is a trace mineral element required for pre-weaned calves so that they can undergo adequate erythropoiesis and growth without becoming anemic. The valence form of Fe that is readily found in ground water is ferrous (**Fe^{2+}**). Research showed that Fe^{2+} is more biologically available than the ferric (**Fe^{3+}**) form in several animal species (Fritz et al., 1970; Van Ravenswaay et al., 2001) and that it can be incorporated into cells. Research also has showed that weaned calves provided dietary Fe (most likely as Fe^{3+}) in excess of requirement (NRC, 2001) had decreased rate of gain, altered Fe metabolism, and increased intestinal permeability for Fe that might influence the calves susceptibility to pathogens (Hansen et al., 2010). Additionally, experimentally increasing concentrations of added Fe^{2+} from 0 to 8 mg/L in drinking water of mid-lactation Holstein cows decreased water consumption (Genther and Beede, 2013).

Water intake drives feed intake in calves (Kertz et al., 1984). It is important that calves in consume adequate volumes of fresh water to remain healthy and maintain adequate rates of gain. Pre-weaned Holstein calves provided the recommended amount of milk replacer of 10% of BW per day (NRC, 2001) can consume 2 L of water daily by 4 wk of age (Kertz et al., 1984; Quigley et al., 2006). Milk replacer and calf starter pellets are typically formulated to contain adequate concentrations of dietary Fe to support normal calf growth and health. Iron consumed from well water as a part of free-choice water consumption and the water used to suspend the milk replacer powder are additional potential sources of Fe intake, most oftentimes of unknown quantities.

The US EPA (2004) indicated that good quality water contains less than 0.3 mg Fe/L. However, this value is based on human taste preference and water aesthetics such as color, odor, and texture; it is not a standard intended for dairy cattle. Socha et al. (2001) reported in a large (n

= 4,072) sampling of well waters from livestock farms across the US a median Fe concentration of 0.10 mg Fe/L; but, with a wide range to a maximum of 123 mg Fe/L.

Dietary Fe concentration should not exceed 1,000 mg Fe/kg DM (NRC, 2001); or else it is suggested that binding capacity of Fe transport protein transferrin can be exceeded and the unbound Fe can create reactive oxygen species leading to oxidative stress in tissues, diarrhea, reduced feed intake, and reduced weight gain (NRC, 2001). However, currently there is no research available on the effects of varying Fe^{2+} concentrations in drinking water on water and starter intake, Fe status indicators, and growth of pre-weaned calves.

The objective of this experiment was to evaluate the effects of varying concentrations of Fe^{2+} in drinking water and milk replacer suspension on whole blood and serum variables of Fe status, drinking water and starter pellet intake, and BW gain of pre-weaned calves. It was hypothesized that Fe status, water and feed intake, and growth would be improved by consuming drinking water and milk replacer with lower concentrations and quantities of Fe^{2+}.

MATERIALS AND METHODS

The Michigan State University (MSU) Institutional Animal Care and Use Committee approved all procedures for the experiment (approval no. 04/14-074-00). The experiment was conducted out-of-doors at the MSU Dairy Teaching and Research Center (DTRC) from July through November, 2014.

Experimental design and animals

Calves entered the experiment at 28 d of age and remained on treatments through d 56 of age. Sixty Holstein calves (25 bulls and 35 heifers) were assigned in a randomized complete block design to one of five water treatments varying in ferrous iron (**Fe^{+2}**) concentrations in twelve blocks. Each block had calves of the same sex and similar age randomly assigned to one of the five Fe^{+2} treatments. Any potential candidate calves were excluded from the experiment if health was compromised in any way at any time before 28 d of age.

Experimental procedures

Calves were born at the DTRC and kept in individual outdoor calf hutches (PolyDome, Litchfield, MN or Calf-Tel Germantown, WI). Before the experiment started they received colostrum and milk replacer (Cow's Match WarmFront® BOV BM DBZ, Land O'Lakes Animal Milk Products Co., Shoreview, MN) (nutrient analysis in Table 3.1), calf starter (Ampli-Calf, Purina Animal Nutrition, St. Louis, MO) (nutrient analysis in Table 3.2), and drinking water from the DTRC well source (laboratory analysis in Table 3.3) according to standard operating procedures.

Standardization period. Calves entered a standardization period at 25 d of age for 3 d. Well water was used as the free-choice drinking water source and to mix with the milk replacer

powder. Milk replacer suspension was prepared as 13.5% DM per manufacturer's instructions using warmed (46^{o}C) well water. Calves were fed 2.4 L of mixed milk replacer suspension at 0800 and 1430 h, and 3.5 L at 2200 h daily. They were given 10 min to consume their allotment and then the bucket was removed. All calves consumed their total allotment at each feeding.

Calves were kept individually in outdoor pens (1.5 m by 2.6 m) with calf hutches bedded with sand (about 5 to 10 cm in depth). A 4.7 L white plastic bucket was placed in a metal bucket-holder on the front of the hutch pen for drinking water except during the 10 min three times a day when milk replacer was fed. A 4.7 L black rubber bucket containing calf starter was positioned in a metal bucket-holder inside the calf hutch.

Experimental period. Procedures used in the standardization period remained the same for the experimental period except that experimental treatments differing in the Fe^{+2} concentrations (0, 2, 4, 8, or 12 mg/L) in the drinking water and water used to prepare the milk replacer suspensions were offered and fed. Treatments with varying Fe^{+2} concentrations were made using ferrous lactate hydrate ($C_6H_{10}FeO_6 \cdot xH_2O$; Sigma-Aldrich, Milwaukee, WI). All drinking water and milk replacer Fe^{2+} treatments were prepared using deionized and demineralized well water treated with a commercial gel type, high capacity, high purity mixed ion exchange resin (Lewatit MN 91 resin, Besco Water Treatment, Inc., Mason, MI; laboratory analysis in Table 3.4) before ferrous lactate was added. Treatments were made iso-lactate using needed amounts of 85% lactic acid (Avantor Performance Materials, Center Valley, PA). In the morning for each day of the experiment the iso-lactate Fe^{+2} water treatments were prepared from previously prepared 500-mL stock solutions added to 19 L buckets of deionized demineralized water. The pH of iso-lactate Fe^{+2} treatment solutions ranged from 5.34 (12 mg Fe^{+2} /L) to 5.91 (0 mg Fe^{+2} /L) over the course of the experiment.

Water and feed sampling

Daily during data collection, a 100-mL sample of water was collected at 0800, 1430, and 2200 h representing each of the following: well water for any calves in the standardization period (Table 3.3); the Fe^{+2} water treatments (Table 3.5); and, the Fe^{+2} water treatments for preparation of milk replacer suspensions (Table 3.6), respectively. Within each category water samples were pooled weekly (100 mL) and acidified with 2 mL of nitric acid (EMD Millipore Corporation, Darmstadt, Germany). The water samples were sent for analysis to Cumberland Valley Analytical Services (Hagerstown, MD). Total recoverable Fe in the acidified samples was determined for each of the 5 Fe^{+2} treatments, and well water samples (collected before deionization and demineralization) were analyzed for calcium carbonate ($CaCO_3$ for hardness), chloride (Cl), sulfate (SO_4), Ca, P, Mg, K, Na, total recoverable Fe, Mn, Zn, and Cu.

Milk replacer and starter feed samples (about 100 g each) were collected each time a new bag was opened. Samples were composited every month and sent for nutrient analysis (Cumberland Valley Analytical Services, Hagerstown, MD). The nutrient analyses are in Tables 3.1 and 3.2, respectively.

Blood sampling

Blood samples (5 mL) for serum were collected using a 21G needle and glass vacutainer tube coated with Silica Act Clot Activator (Becton Dickenson, Franklin Lakes, NJ) via the jugular vein between 0745 to 0800 h on d 0 (prior to calves entering the experimental period and receiving treatments) and at 2 and 4 wk of the experiment and prior to taking body measurements (described below). The blood sample was allowed to clot at room temperature for 30 min and then centrifuged at 5°C and 1,200 × *g* for 15 min. Serum was collected and transferred to two 1.5-mL microfuge tubes and stored at -23°C until analysis. Serum was analyzed for Ca, P, Mg,

Fe, total iron-binding capacity (**TIBC**), and total iron-binding saturation (**TIBS**) at the Kansas State University Veterinary Diagnostic Laboratory (Manhattan, KS). Serum Fe and TIBC were determined by spectrophotometric measurement.

Total iron-binding capacity is a way to assess serum Fe deficiency or overload in the blood. It is an indirect measure of Fe bound to transferrin. The transferrin-bound Fe is referred to as serum Fe. The serum Fe can be divided by TIBC and multiplied by 100 to determine the percentage of Fe saturation (TIBS). If TIBC exceeds serum Fe, then there is no unbound Fe present in the serum and thus no assumed (presumed ← is this a better word?) toxicity.

A second 5-mL whole blood sample was collected at the same time and days as the serum using a vacutainer tube coated with ethylenediaminetetraacetic acid (**EDTA**); it was sent immediately for Complete Blood Count analyses at the MSU Diagnostic Center for Population and Animal Health (East Lansing, MI). Variables and analytes assayed included: hemolysis, lipemia, icterus, total protein, red blood cell count, hemoglobin, spun hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, corpuscular hemoglobin concentration mean, red blood cell distribution width, platelet count, mean platelet volume, and white blood cell count (lymphocyte, monocyte, eosinophil, neutrophil (segmented and banded), and basophil.

Growth measurements

Body weight, length, hearth girth, hip width, hip height, and wither height were measured at 26, 27; 41, 42; and, 55 and 56 d of age immediately after taking blood samples. Body length was measured from the scapula to the tuber ischii, heart girth at the olecranon, and hip width from the points of the tuber coxae all with a measuring tape (Nugent et al., 1991). Wither height

and hip height were measured with a measuring stick (Nasco, Fort Atkinson, WI). These multiple body measurements were made to complement BW data taken at the same time.

Statistical analysis

Data were evaluated for normality of residuals and homogeneity of variance. Daily DMI data were normalized with a square root transformation and daily water intake data were normalized with a natural log-transformation. Results for statistical analyses of these variables were transformed back for presentation. Individual calf within block was the experimental unit. Data were analyzed by ANOVA using the MIXED procedure of SAS (SAS Institute Inc., Cary, NC). Daily DMI and water intake were averaged over 1-wk periods for each of the 4 wk of the experiment. Dry matter intake and water intake data were then analyzed using a model that included the covariate (pre-experiment measurement of variable during the standardization period d 25 to 27): $y_{ijklm} = \mu + COV + S_i + A(C)_j + W_k + X_l + SW_{jk} + SX_{jl} + WX_{kl} + \varepsilon_{ijkl}$; where: μ = overall mean, S_i = fixed effect of sex, $A(C_{)j}$ = random effect of calf within block (j = 1 to 60), W_k = fixed effect of week (k = 1 to 4, repeated measure), X_l = fixed effect of treatment (l = 0, 2, 4, 8, or 12 mg Fe/L), SW_{ik} = interaction of sex and week, SX_{jl} = interaction of sex and treatment, WX_{kl} = interaction of week and sex, and ε_{ijkl} = residual error. Auto-regressive order 1 was used as the covariance structure in the repeated measures analysis and calf (block × sex × treatment) was the subject.

Body measurements of weight and heart girth were averaged for the two consecutive days at 2-wk intervals (26 and 27; 42 and 43; and, 55 and 56 d of age) and the averages represented d 0, and 2 and 4 wk of the experimental period. Blood serum, whole blood, and body measurement data (2 and 4 wk) were analyzed by ANOVA with a model that included the

covariate (pre-experiment measurement of variable on d 0): $y_{ijklm} = \mu + Cov + S_i + A(C)_j + W_k + X_l + SW_{jk} + SX_{jl} + WX_{kl} + \varepsilon_{ijkl}$; where Cov = d 0 data, $A(C)_j$ = random effect of calf within block (j = 1 to 60) where: μ = overall mean, S_i = fixed effect of sex, W_k = fixed effect of week of experiment (k = 2 or 4, repeated measure), X_l = fixed effect of treatment (l = 0, 2, 4, 8, or 12 mg Fe/L), SW_{ik} = interaction of sex and week, SX_{jl} = interaction of sex and treatment, WX_{kl} = interaction of week and sex, and ε_{ijkl} = residual error. Average daily gain (ADG) was analyzed for d 0 to 2 wk, 2 to 4 wk, and d 0 to 4 wk by ANOVA using the model: $y_{ijklm} = \mu + S_i + A(C)_j + X_l + SX_{jl} + \varepsilon_{ijkl}$; where $A(C)_j$ = random effect of calf within block (j = 1 to 60) where: μ = overall mean, S_i = fixed effect of sex, $A(C)_j$ = random effect of calf within block (j = 1 to 60), X_l = fixed effect of treatment (l = 0, 2, 4, 8, or 12 mg Fe/L), SX_{jl} = interaction of sex and treatment, and ε_{ijkl} = residual error. For all ANOVA analyses, main effect results were considered significant if $P < 0.05$ and trends or tendencies if $P > 0.05$, but $P < 0.10$, and interactions were considered significant if $P < 0.10$ and trends or tendencies if $P < 0.15$.

RESULTS AND DISCUSSION

The purpose of this experiment was to evaluate the effects of varying concentrations of Fe^{2+} (0, 2, 4, 8 and 12 mg Fe^{2+}/ L) in drinking water and milk replacer suspension on blood indicators of Fe status, water and starter intake and growth performance.

Indicators of iron status

Serum Fe. Serum Fe is a measure of Fe bound to transferrin in the blood. Transferrin is the protein that transports Fe in blood. It is estimated that one-third of transferrin is bound by Fe (De Jong et al., 1990). Overall, serum Fe concentrations (ranging from about 220 to 330 µg/dL) were affected by the Fe^{2+} concentration of treatments ($P < 0.04$, SEM = 18.0; pooled across week and sex). There was a linear increase due to treatment Fe^{2+} across the whole experiment ($P = 0.02$; Table 3.4). There also was a treatment by week interaction ($P < 0.05$, SEM = 25.8; Figure 3.1). At wk 2 there was a relatively wide range in serum Fe values among treatments with calves in the 12 and 8 mg Fe^{2+}/L treatments having the greatest values; but, by wk 4 this spread narrowed considerably (Figure 3.1). When tested separately by week of sampling at wk 2 there was a linear effect of treatment ($P < 0.01$; pooled across sex and week); however, by wk 4 there was no effect ($P > 0.05$). There also were no main effects of wk, sex, or interactions of treatment by sex, week by sex, or treatment by week by sex on serum Fe ($P > 0.05$). Serum Fe concentrations for calves in all treatments remained greater than 250 µg/dL on average and were greater than reported by Piccione et al. (2010) for healthy calves; or, calves provided high dietary Fe in feed (Hansen et al., 2010). Serum Fe concentrations for calves in the current experiment were similar to values for calves provided excess Fe supplied from ferrous sulfate in milk replacer by Jenkins and Hidiroglou (1987). Ferrous iron is more biologically available than ferric iron in feeds (Fritz et al., 1970; Van Ravenswaay et al., 2001). In the current experiment it

appears that sufficient Fe of high biological availability was provided via both the milk replacer suspension and drinking water. A potential explanation is that the absorption coefficient of Fe^{2+} changed over this time. The amount of Fe that is absorbed by a calf is most dependent on how much Fe a calf is consuming, followed by requirements and age of a calf (NRC, 2001). A calf will absorb less Fe from its diet even if it has not met its biological requirements for hemoglobin and growth. This coefficient can range from about 40 to 70% absorption, depending on the amount of Fe consumed by calves (NRC, 2001). The decrease in serum Fe observed at wk 4 for calves provided the greatest Fe treatments in this experiment suggests a dietary threshold for Fe was consumed and less Fe was absorbed by wk 4. Results of this experiment do not suggest that toxicity occurred with any of the treatments provided to calves from 28 to 56 d of age.

Other uses for Fe within the system that could account for a decrease of Fe observed as serum Fe include incorporation into hemoglobin, myoglobin, and the liver (as hemociderin), or excretion as part of feces (NRC, 2001). To better understand how Fe is incorporated into other parts of the body, an assay for its storage protein, ferratin, would be required. This assay is specie specific and unfortunately there is not one available for bovine ferratin at this time.

***Total iron-binding capacity*.** The total iron-binding capacity (**TIBC**) is a measure of how much Fe can be bound by transferrin within the blood. It is an indirect measure of transferrin and TIBC and serum Fe typically are inverse to one another; as the concentration of Fe decreases in the blood and less is bound to transferrin, the greater number of open binding spots on transferrin are available and thus a higher TIBC. The TIBC has a normal reference interval and if TIBC exceeds this interval, then this would be suggestive of Fe deficiency and anemia. Similarly, if TIBC is below this interval, it would be suggestive of Fe overload and risk of Fe toxicity. In the current experiment, there was no effect of Fe^{2+} treatment on TIBC, but there was an effect of wk of experiment in which TIBC increased from 614 to 632 μg/dL by wk 4 ($P = 0.04$, SEM = 9.2).

An inverse relationship of serum Fe to TIBC was observed with an overall decrease in serum Fe between wk 2 and 4, and an increase in TIBC over this time. No main effects of treatment, sex, or two- and three-way interactions were detected for TIBC ($P > 0.05$).

Total iron-binding saturation. The TIBS is a calculation of serum Fe divided by TIBC, multiplied by 100. In cases of Fe toxicity transferrin binding becomes fully saturated and unbound Fe in the blood becomes toxic (Moser et al., 1994). Unbound Fe increases free radicals causing oxidative stress, which can increase incidence of disease in animals (Albretson, 2006). As Fe^{2+} concentrations increased in drinking water and milk replacer suspension of experimental treatments TIBS increased ($P = 0.02$, SEM = 2.6; Figure 3.2). Calves on 8 and 12 mg Fe/L had TIBS values greater than 50%, whereas calves on 0, 2, and 4 mg Fe/L had values below 50%. There were no main effects of wk of experiment or sex on TIBS. There was an overall linear effect of Fe^{2+} treatment ($P = 0.01$) on TIBS over the entire experiment of 4 wk. This was heavily influenced by TIBS values at wk 2 ($P < 0.01$, linear effect of treatment), but not at wk 4 ($P > 0.05$). There also was a treatment by wk interaction ($P = 0.03$, SEM = 3.6; Figure 3.3). Calves on treatments 8 and 12 mg Fe/L had the greatest saturation percentages at 2 wk followed by 2, 0, and 4 mg Fe/L, respectively; and, there was a greater range in values among treatments at 2 wk, but the range narrowed considerably by wk 4. Also, the TIBS values are similar in relative ranking among treatments over the entire 4-wk experiment compared with the serum Fe concentrations (Figure 3.1). This is expected as TIBS is a direct calculation of serum Fe. The TIBS values in this experiment were greater for all treatments than those reported by Mohri et al. (2004) who supplemented calves daily with a greater quantity of Fe^{2+} than the treatments provided in the current experiment. It is unknown if there is an ideal TIBS for calves that is less than 100%. The literature for humans suggests a TIBS greater than 50% might be indicative of Fe overload (Mainous et al., 2004). Calves provided 8 and 12 mg Fe/L treatments had a TIBS of

54% and 52%, respectively, at wk 2 which subsequently dropped to 47% for both treatments by wk 4. This decrease in TIBS is associated with the serum Fe concentrations that had a trend for a week by treatment interaction ($P < 0.03$, SEM = 3.6) with a decrease in serum Fe between 2 and 4 wk as well. Interestingly, the 0 mg Fe/L treatment did not have the lowest TIBS and it increased to the greatest saturation percentage (50% TIBS) at wk 4. The reason for this interaction of treatment and wk is unclear. Serum Fe and TIBS values were expected to continue to increase as intake of Fe^{2+} continued to increase over this time. Because TIBS is a direct calculation from serum Fe, the decrease in TIBS at wk 4 could be caused by the same events that could cause a decrease in serum Fe; a decrease in absorption coefficient of Fe, incorporation of Fe into hemoglobin, incorporation of Fe into myoglobin, and excreted Fe as part of feces. The increase in TIBS at wk 4 for calves on 0 and 4 mg Fe/L treatments could be due to a combination of increase in intake of Fe from starter and water as well as a change in absorption coefficient.

Calf performance

Drinking water intake. Ferrous iron treatments or sex of calf did not affect free drinking water intake over the course of the experiment ($P > 0.05$). Water intake increased as week of the experiment increased pooled across treatment and sex (Figure 3.5; $P < 0.01$, SEM = 0.07). Water intake increased from 1.4 L/d in wk 1 (28 to 35 d of age) to 1.8 L/d by wk 4 (Figure 3.5). This increase was expected as water intake typically increases as calves grow. The response in water intake was similar to that found in other research with similar sized pre-weaned Holstein calves (Kertz et al., 1984; Huuskonen et al., 2011). An experiment evaluating the effects of ferrous lactate on lactating cows showed a decrease in the consumption of drinking water with 8 mg Fe/L compared with 0 or 4 mg Fe/L when given a choice (Genther and Beede, 2013). Calves in

this experiment did not decrease water consumption with 8 or 12 mg Fe/L treatments compared with those offered 0, 2, or 4 mg Fe/L. Calves were closely monitored for signs of health issues throughout the experiment. At no time were there refusals of milk replacer suspension made with any of the treatment waters and no calves were removed from the experiment due to complete refusal of drinking water or health concerns.

Starter pellet intake. There was no effect of experimental treatment or sex on starter pellet intake over the course of the 4-wk experiment ($P > 0.05$). Starter pellet intake increased as week of experiment increased ($P < 0.01$, SEM = 0.04; Figure 3.6). Starter intake increased from 0.35 kg/d per calf in the first week of the experiment (28 to 35 d of age) to 1.24 kg/d per calf by wk 4. This increase was expected because calves normally increase dry feed consumption as they grow. The amount of starter consumed was similar to other research (Jasper and Weary, 2002; Huuskonen et al., 2011) with similar sized Holstein calves consuming milk replacer suspension provided at the minimum the NRC (2001) recommendation of 10% of the calf's BW.

Daily Fe consumption. The NRC (2001) recommends a daily Fe intake based on age of calf and dry matter consumed. This recommendation is 150 mg Fe/kg DM. This equates to 187 to 306 mg Fe/d for calves on this experiment between 28 and 56 d of age. There was no effect of experimental treatment or sex of calf on dietary Fe intake from starter pellets ($P > 0.05$). Dietary Fe intake from the starter pellets increased each successive week of the experiment ($P < 0.01$, SEM = 0.59; Table 3.5). This was due to increased intake of starter pellets over this time. Calves received 122 mg Fe/kg DMI of starter. The Fe intake from free drinking water increased as concentration of Fe in water increased ($P < 0.01$, SEM = 0.18; Table 3.5) and also as wk of experiment increased ($P < 0.01$, SEM = 0.13). Calves consumed between 0 and 24 mg Fe/d in free drinking water depending on the treatment. The increase in Fe intake from water was due to increased consumption of water as calves grew. There was no effect of sex on Fe intake from

water. The mg Fe/d from milk replacer suspension remained constant within each Fe treatment because the daily allotment was constant for each day of the experiment. Calves received 137 mg Fe/d from milk replacer powder and between 0 and 86 mg Fe/d from the milk replacer suspension water, depending on treatment.

Total mg Fe/d is a sum of mg Fe consumed as starter pellets, milk replacer powder, milk replacer suspension water treatment, and free drinking water treatment. Total mg Fe/d increased as Fe treatment increased ($P < 0.01$, SEM = 0.07) and as week of experiment increased ($P < 0.01$, SEM = 0.04). There was no effect of sex on mg Fe/d. Total mg Fe/d is shown in Table 3.6. These intakes were calculated as a percentage of the recommended mg Fe/d for calves based on their DMI (150 mg Fe/kg DM; NRC, 2001). Interestingly, calves consumed as much as 7% above the recommended daily intake when provided 0 mg Fe/L treatment. This Fe would have solely come from milk replacer powder and starter intake. The NRC (2001) recommendation is a rough estimate of Fe requirements and calves on this experiment would have to consume more than 1000 mg Fe/d for toxic levels of Fe (NRC, 2001). Calves consumed more than 120% of their recommended daily intake when provided treatments 4, 8 or 12 mg Fe/L each week. Calves provided 12 mg Fe/L consumed over 150% their recommended daily Fe each week. The differences in daily Fe intake by wk and treatment are shown in Table 3.7. Because DMI increases over time, the percentage of Fe above the recommended daily intake (as based on DMI) also changes.

There is a large range for daily Fe intake between recommended and toxic mg Fe/d, and these NRC recommendations are based on research using less bioavailable Fe^{3+}. Percentage of Fe^{2+} as total Fe intake was calculated assuming that Fe^{2+} only came from free drinking water treatment and milk replacer suspension water treatment. The Fe from starter pellets and milk replacer powder is assumed to be of Fe^{3+}. On average, calves provided 0, 2, 4, 8, or 12 mg Fe/L

treatments consumed 0, 5, 9, 17, or 22% of their Fe, respectively, as Fe^{2+} (Table 3.8). Considering these calves were consuming daily mg Fe above the recommended amount, the percentage of that as highly biologically available Fe^{2+} is worth note. Because serum Fe did not exceed the reference range and TIBS did not reach above 52% throughout the experiment, it is unclear if there is a threshold for daily Fe^{2+} or if the absorption coefficient of Fe^{2+} decreased as the proportion of Fe^{2+} increased in the diet.

Body weight, average daily gain, and growth measurements. Iron treatment affected BW ($P = 0.01$; Figure 3.7) and a trend for an effect of treatment on ADG ($P = 0.08$, SEM = 0.06; Table 3.9). However, there was no effect of treatment on heart girth, body length, hip width, hip height, or wither height. There was an effect of wk on BW ($P < 0.01$, SEM = 0.73) with a trend for a quadratic effect of treatment in results in wk 4 ($P = 0.08$) and an effect of wk on heart girth ($P < 0.01$), body length ($P < 0.01$), hip height ($P = 0.04$), and wither height ($P = 0.04$). However, there was no effect of wk on ADG or hip width. There was an effect of sex on ADG ($P < 0.02$, SEM = 0.04) and heart girth ($P = 0.01$) with bulls having a greater ADG (1.16 kg/d) than heifers (0.99 kg/d) and greater heart girth. There was no effect of sex on BW, body length, hip width, hip height, or wither height; however, there was a trend for an interaction of sex by wk on BW ($P = 0.07$; Figure 3.8) with bull and heifer calves weighing approximately the same at wk 2, but bulls weighing more by wk 4. There was a trend for an interaction of treatment by sex on heart girth ($P = 0.08$).

In the current experiment the ADG of pre-weaned heifer and bull calves was greater than averages reported by Jasper and Weary (2002), Quigley et al. (2006), and Huuskonen et al. (2011) even though starter intakes were quite similar among all the experiments. This is likely due to a larger quantity of milk replacer suspension provided in this experiment (8.3 L/d, 13% DM). Calves provided the 4 mg Fe/L treatment were of greater BW on average than calves

provided the other 4 treatments and these calves also had greatest ADG (1.2 kg/d). These calves did consume more starter compared with the average of the other treatment groups (0.85 kg vs. 0.66 kg, respectively), though this DMI difference between treatments was not significant and does not explain the difference in BW among treatments. Calves provided 2 mg Fe/L had the least ADG (0.97 kg/d). Differences in ADG among the remaining three treatments were less than 0.04 kg/d. The reason for this difference is unknown, however this difference in ADG between treatments is biologically minimal and is not thought to be of substantial consequence. While treatment affected BW gain, it did not have the same pronounced effect on ADG. This difference is likely due to a covariate being used as part of the analysis for body weight gain.

Measuring specific growth variables are important for monitoring replacement calf growth benchmarks. Heart girth was expected to increase with progression of the experiment due to calf growth, and since bull calves were larger at the start of the experiment, they were expected to remain larger throughout the course of this experiment. The differences in heart girth corroborate the BW and ADG findings for wk and sex differences. However, the lack of treatment effects on any of the growth variables greatly supports there being no biological significance of Fe^{2+} treatment on the growth of calves of this age, even though BW was affected by treatment. Changes in BW of calves are due to fat and muscle deposition and bone growth. The measurements taken in this study do not specifically identify whether it is fat, muscle, or bone growth that is occurring. Therefore, while the increase in BW of calves provided the 4 mg Fe/L treatment is interesting, the lack of effect of this treatment on other growth variables suggests that the overall biological effects of this treatment on growth are minimal. Mean values of body measurements by wk are provided in Table 3.9 for each bulls and heifers.

Serum macrominerals

Calcium. Reference intervals for serum Ca is 10.7 to 11.2 mg/dL (Egli and Blum, 1998) .Most serum Ca values were within range of those reported for healthy calves of this age. There was an effect of treatment on serum Ca ($P < 0.01$, SEM = 0.12) with a quadratic effect of treatment ($P < 0.01$; Figure 3.9). This quadratic effect was mainly due to responses to treatment in wk 2 ($P = 0.02$, SEM = 0.10; Figure 3.10). The reason for this quadratic effect of treatment is not understood. The 0 mg Fe/L treatment had a serum Ca of 11.5 mg/dL that was slightly outside the reference range, but not thought to be of biological significance. Research has not been done to evaluate the potential effects dietary ferrous Fe has on calf serum Ca, although some human studies showed increased dietary Ca decreased Fe absorption (Lönnerdal, 2010). An increase in dietary Ca relative to dietary Fe is not a factor in this case as starter pellet intakes were similar among treatment groups and daily milk replacer amount was the same for all calves thus keeping Ca intake constant across Fe^{2+} treatments. Age-related changes in serum Ca in calves between 28 and 56 d of age were reported by Mohri et al. (2007). Normal biological changes during this time might influence the utilization of Fe^{2+} and Ca creating the quadratic effect seen in this experiment, however, the reason for this cannot be explained based on the results available from the current experiment. There was no effect of wk or sex on serum Ca.

M*agnesium and phosphorus.* Blood serum Mg (reference 1.5 – 2.4 mg/dL) and P (reference P 7.4 – 9.3 mg/dL) were within normal physiological ranges (Egli and Blum 1998). There was no effect of treatment or sex on Mg or P concentrations. Serum P tended to increase with increasing time on experiment from 7.95 mg/dL at wk 2 to 8.19 mg/dL at wk 4 ($P = 0.08$, SEM = 0.10). There was a trend for treatment by sex interaction ($P = 0.05$; Figure 3.11). Bulls tended to have greater serum P concentrations for all treatments except for the 2 mg Fe/L treatment. The reason for this is not known. There was a trend for wk by sex interaction ($P =$

0.08; Figure 3.12) with serum P of heifers increasing over time whereas that of bulls remained relatively constant over time. Serum P was within the range reported by Mohri et al. (2007).

Complete Blood Count Variables

Complete blood count was taken to provide an overall picture of calf health using measurements of red blood cells (size, shape, and volume) and of white blood cells. This can be used to identify cases of anemia, blood loss, and heath crises that may be attributed to toxicity.

Hematocrit. Spun hematocrit (Hct) values in this experiment are within range (reference 25 – 40%; Knowles et al., 2000; Egli and Blum, 1998) of calves of this age group as reported by others. Hematocrit was measured to indicate change in red blood cell production relative to change in treatment. There was an overall effect of treatment ($P < 0.01$; Figure 3.13) and sex ($P = 0.03$) on spun Hct. The response across the whole experiment was curvilinear ($P < 0.01$), influenced most by values from the wk 4 sampling ($P < 0.01$ quadratic; Figure 3.14), but less by a similar quadratic effect of treatment at wk 2 ($P = 0.059$, SEM = 0.31). The greatest hematocrit values were for calves provided 0 or 12 mg Fe/L treatments. Heifers had a slightly greater hematocrit (38%) than bulls (37%; $P < 0.03$, SEM = 0.36); the biological significance of this is not known. A greater hematocrit might be an indication of dehydration indicating that calves were not consuming enough water for their biological needs and a lower hematocrit would indicate red blood cell production deficiency, destruction, or loss. Adequate hematocrit values in this experiment support that these issues were not present even though hematocrit varied as much as 2.4% among treatments.

Hemoglobin. Hemoglobin can be another indicator of Fe status. If hemoglobin concentration decreases below the normal range (reference 8 to 12 g/dL; Egli and Blum, 1998; Brun-Hansen et al. 2006) it can be a sign of anemia, or if hemoglobin concentration increases

above the normal range it can be a sign of abnormally high red blood cell production. Hemoglobin values in the current experiment were slightly above the reference range. Anemia or abnormal red cell production are most likely not a concern for calves in this experiment.

However, there was an overall main effect of Fe^{2+} treatment ($P = 0.01$) on hemoglobin concentration; a curvilinear response over the 4-wk experiment ($P < 0.01$; Figure 3.4). Much of this effect was due to the response to treatment at wk-4 blood sampling ($P < 0.01$); there was not a similar curvilinear effect detected at the wk-2 sampling. There was a trend for an effect of week of experiment on hemoglobin concentration ($P = 0.09$). There was no effect of sex on hemoglobin concentration; however, there was trend for a treatment by sex interaction ($P = 0.07$). Hemoglobin tended to increase slightly between 2 (12.3 g/dL) and 4 wk (12.5 g/dL; SEM = 0.13), overall ($P = 0.09$); and, heifers tended to have greater hemoglobin concentrations than bulls ($P = 0.07$) for 0, 2, and 8 mg Fe/L treatments. Hemoglobin concentrations for heifers were 12.9, 12.6, 12.3, 12.4, and 12.7 g/dL for 0, 2, 4, 8, and 12 mg Fe/L treatments, respectively; whereas, for bulls hemoglobin concentrations were 12.1, 12.0, 12.3, 11.6, and 12.9 g/dL, for 0, 2, 4, 8, and 12 mg Fe/L treatments, respectively.

It is unknown if the differences in hemoglobin concentrations among treatments in this experiment are biologically significant. There was a 6% difference between the lowest hemoglobin concentration for the 8 mg Fe/L treatment (12.0 g/dL; SEM = 0.18) and the greatest hemoglobin concentration for the 12 mg Fe/L treatment (12.8 g/dL; Figure 3.4); but, all were values within reference ranges of healthy calves of similar ages. Similar values were reported by others (Mohri et al., 2007; Ježek et al., 2011).

Iron is an important component of hemoglobin and it is possible that dietary Fe is related to hemoglobin concentration. Hansen et al. (2010) found no change in hemoglobin concentration

of calves provided about 750 mg Fe/head per d; whereas, calves in the current experiment consumed between about 270 to 400 mg Fe/head per d from iron treatments in drinking water and milk replacer suspension, plus that starter consumption. The difference in these results could be due to the different iron sources used and differing bioavailability. Hemoglobin is tightly controlled within the body and the range seen in this study is not thought to have a positive or negative impact on the calves.

Mean corpuscular hemoglobin. The normal range of corpuscular hemoglobin (**MCH;** average mass of hemoglobin per red blood cell) is 10 - 13 pg (Egli and Blum, 1998; Jezek et al. 2011). It was measured in the current experiment to assess how Fe^{2+} treatment might affect hemoglobin production. There was an effect of treatment on MCH ($P = 0.05$; Figure 3.16). There was no effect of wk; however, there was effect of sex of calf on mean MCH ($P = 0.04$). Mean corpuscular hemoglobin was greater among bull calves (12.4 pg, SEM = 0.06) than heifer calves (12.2 pg, SEM = 0.06). There was an effect of treatment with an overall cubic effect ($P < 0.01$; Figure 3.16) on MCH, a trend for a cubic effect of treatment at wk 2 ($P = 0.06$; Figure 3.16), and an effect of treatment at wk 4 cubic ($P = 0.04$; Figure 3.16). The MCH was within range of reported by Jezek et al. (2011) and greater than that reported by Knowles et al. (2000) and Mohri et al. (2007) for similarly aged calves. The MCH is a relationship with hemoglobin, and considering there was a quadratic effect of treatment on overall hemoglobin concentration, observing a change in average hemoglobin per red blood cell is expected. However, similar patterns of change were not noted between hemoglobin concentration and MCH. The 0 and 8 mg Fe/L treatments had the lowest MCH, and 4 mg Fe/L treatment had the greatest MCH. Clinically, MCH is considered with mean corpuscular volume (**MCV**) when determining biological significance. This is because MCV influences the amount of hemoglobin that can be present within each red blood cell and thus MCH and MCV should increase or decrease in a similar

fashion. There was no effect of treatment or sex on MCV. There was a trend for a decrease in MCV ($P = 0.0914$) between 2 and 4 wk (35.4 and 34.8 fL, respectively; SEM = 0.34). This suggests that while cellular volume did not change for calves among the treatments, the mass of hemoglobin did. The reason for this is unclear.

The normal range of corpuscular hemoglobin concentration (**MCHC;**) is 32 - 36 g/dL (Jezek et al., 2011; Brun-Hansen et al. (2006). The MCHC is average weight of hemoglobin per hematocrit volume and is a way to assess hemoglobin and hematocrit are changing in proportion to one another. There was no effect of treatment on MCHC. There was an effect of wk on MCHC ($P < 0.01$) with MCHC increasing from 34.2 to 34.9 g/dL (SEM = 0.16). There was no effect of sex on mean corpuscular hemoglobin concentration. An increase in MCHC indicated an increase in hemoglobin or a decrease in hematocrit. As stated previously, there was change in hematocrit over this time although there was a trend for hemoglobin to increase over this time ($P = 0.09$).

Cell hemoglobin concentration mean (**CHCM**) is a measure of hemoglobin within intact red blood cells and is a more accurate measurement of hemoglobin concentration compared with MCHC. There is no specified range specifically for calves, but it should be close to the reported MCHC reference range of 32 – 36 g/dL. There was no effect of treatment on cell hemoglobin concentration mean (**CHCM**). There was an effect of wk on CHCM ($P < 0.01$) with cell hemoglobin concentration mean increasing 33.0 g/dL to 33.5 g/dL (SEM = 0.11). There was no effect of sex on CHCM.

Red blood cell distribution. The normal range of red blood cell distribution width is 20 – 25 % (Brun-Hansen et al. 2006). Red blood cell distribution width is an indicator of red blood cell production abnormalities. The larger the distribution width from normal, the more variable

sizes of red blood cells there are. This can indicate rapid production of new red blood cells. Red blood cell distribution width can be used in conjunction with MCV to determine cause of anemia. There was a treatment effect on red blood cell distribution width percentage ($P = 0.04$). There was no effect of wk or sex on red blood cell distribution width percentage. There was an overall quadratic effect of treatment ($P = 0.01$; Figure 3.17) with a trend for a quadratic effect of treatment at wk 2 ($P = 0.09$) and wk 4 ($P = 0.07$; Figure 3.18). Although there was a treatment difference, RDW remained within reference ranges and calves did not have destruction, over production, or loss of red blood cells.

Research to date shows conflicting results of hematological variables of young calves. Much of these differences might be associated with breed, diet, and health along with individual biological variation (Knowles et al., 2000; Brun-Hansen et al., 2006). Hemoglobin, mean corpuscular hemoglobin concentration and MCV have been reported to decrease between birth and 2 mo of age (Knowles et al., 2000; Brun-Hansen et al., 2006) and there are differences in these variables when low and high white blood cell counts are considered (Knowles et al., 2000).

Platelet count. The normal range of platelet count is 400,000 – 1,000,000/μL (Egli and Blum, 1998; Brun-Hansen et al. 2006). A dramatic decrease in platelets indicates they are being used in the coagulation pathway, they are being destroyed by the immune system, or they are being lost with mass blood loss. There was an effect of treatment ($P = 0.01$) and wk ($P = 0.01$) on platelet count. There was no effect of sex on platelet count. There was a linear effect of treatment on platelet count ($P = 0.03$; Figure 3.19) with a trend for a linear effect in wk 2 results ($P = 0.08$; Figure 3.20). Platelet count also decreased by week. Platelet count decreased as Fe treatment increased. The 12 mg Fe/L treatment had a 14% lower platelet count than the average of the other four treatments although no calves were thrombocytopenic. All platelet values were within range of those reported for healthy calves of that age (Brun-Hansen et al. 2006; Ježek et

al., 2011). These differences in platelet values are likely a confounding result of biological variation with growing calves considering there was not a treatment by week interaction.

Mean platelet volume. The MPV is an indication of platelet production or destruction and is used in conjunction with platelet count. There is no specified range for MPV. There was a trend for a treatment effect on mean platelet volume (**MPV**; $P = 0.09$). There was no effect of wk or sex on MPV. There was a trend for wk by sex interaction ($P = 0.08$). The MPV tended to increase with increasing Fe treatment and heifer calves tended to increase over time while bull calves tended to decrease over time. A large MPV indicates an increase in platelet production while a large MPV and decreased platelet count would indicate large quantities of platelet destruction. While there were differences in platelet count between treatments and a trend for treatment effect on MPV, these are not thought to be biologically significant since platelet count remained within reported reference ranges.

Monocytes. The normal range of monocyte count is 200 – 800/μL (Knowles et al. 2000; Brun-Hansen et al. 2006). Monocytes were assessed as a measure of health of the calves in conjunction with platelet count and white blood cell count. There was an effect of treatment on monocyte concentration ($P = 0.03$). There was no effect of wk or sex on monocytes. There was an overall quadratic effect of treatment of treatment on monocytes ($P < 0.01$; Figure 3.21) with a quadratic effect of treatment at wk 2 ($P < 0.01$; Figure 3.22). Monocyte count at 2 wk was below 400/µL for the 4 mg Fe/L treatment while all other treatments had counts above 400/µL. There was no effect of treatment, wk, or sex on lymphocytes. There was no effect of treatment, week, or sex on white blood cells. An increase in white blood cell count, lymphocyte count, or monocyte count would indicate a disease process. Whereas, monocytes increased the concentration was below the threshold (800/µL) indicative of disease. No calves had to be removed from this experiment for disease and none had to be treated for disease while on the

experiment. Brun-Hansen et al. (2006) reported increase in monocytes of healthy calves between 2 and 4 wk of age while Knowles et al. (2002) reported a decrease in monocyte count. Differences in these studies were attributed to manual versus automated counting of white blood cells. Manual counts of white blood cells were used in this experiment.

CONCLUSIONS

In conclusion, greater concentrations of Fe^{2+} in drinking water and water used for milk replacer suspension affected iron status indicators of pre-weaned calves. The increase in serum Fe and TIBS with increasing Fe^{2+} suggest that calves can consume enough of Fe^{2+} in water to affect these variables. However, the amount of Fe^{2+} consumed in this experiment from drinking water and milk replacer suspension were not enough to saturate transferrin 100% and thus these increases in serum Fe and TIBS are not thought to be detrimental to the calf by the way of oxidative stress from unbound Fe. Outward signs of Fe toxicity such as significant decrease in weight gain, diarrhea, and decreased food and water intake were not observed. However, other measures of total body Fe such as ferratin stored in body cells and measures of oxidative stress such as increased intestinal impermeability were not measured and may aid in identifying Fe overload. Other blood variables that were affected by treatment such as hemoglobin, hematocrit, MCH, and red blood cell distribution width remained within reported values by others. These variables are reported to rise and fall throughout the first 3 months of a calf's life by other researchers. Because these variables change greatly by wk for young growing calves, it is difficult to say whether or not the treatment differences observed in this experiment are biologically detrimental to these processes. Drinking water and starter pellet intake increased by week, as expected with growing calves, and growth was not substantially affected by Fe^{2+} treatment. Overall, the Fe^{2+} treatments in this experiment did not detrimentally affect pre-weaned calves between 28 and 56 d of age. Future research should evaluate the effects of Fe^{2+} in drinking water on pre-weaned calves in the first month of life, and after weaning when they are consuming more water and their hematological variables begin to narrow within an adult range.

APPENDIX B

TABLES AND FIGURES REFERENCED IN CHAPTER 3

TABLES

Table 3.1. Nutrient composition of milk replacer[1] and starter pellet[2] used in standardization period and experiment 3[3]

	Milk Replacer			Starter	
Component	**Percent**	**± SD**		**Percent**	**± SD**
Moisture	6.5	1.76		12.20	0.27
DM	93.5	1.76		87.80	0.27
Mcal/kg of DM					
NEm				1.65	0.01
NEg				1.06	0.01
Percent of DM					
CP	27.9	0.92		22.5	0.40
Ash	11.00	0.15		8.21	0.30
Ca	1.01	0.14		1.01	0.19
P	0.93	0.12		0.65	0.12
Mg	0.16	0.02		0.32	0.06
K	2.59	0.36		1.46	0.25
Na	1.18	0.16		0.51	0.06
mg/kg of DM					
Fe	122	14.70		198	9.75
Mn	41	4.99		163	51.8
Zn	60	15.61		221	72.62
Cu	24	18.21		52	16.38

[1]Cow's Match WarmFront® BOV BM DBZ, Land O'Lakes Animal Milk Products Co., Shoreview, MN.
[2]Ampli-Calf, Purina Animal Nutrition, St. Louis, MO.
[3]Average of composite samples taken each month for 5 mo of experiment.

Table 3.2. Analyte composition of ground (well) water before and after demineralization and deionization for experiment 3[1]

	Before			After	
Component	**mg/L**	**± SD**		**mg/L**	**± SD**
Hardness, ppm $CaCO_3$	420.40	9.42		4.00	0.00
TDS[2]	665.60	183.40		8.00	0.00
Cl	120.00	65.57		18.00	11.31
SO_4	93.44	7.40		< 10	NA
Ca	107.80	6.14		< 1.00	NA
P	< 0.10	NA[3]		< 0.10	NA

Table 3.2 (cont'd)

Mg	32.96	0.64		< 1.00	NA
K	1.98	0.44		< 1.00	NA
Na	8.61	2.12		< 1.00	NA
Fe	0.68	0.41		< 0.05	NA
Mn	0.09	0.01		< 0.05	NA
Zn	0.43	0.23		< 0.01	NA
Cu	0.06	0.05		< 0.01	NA

[1]Average of weekly composite samples taken over 5 mo.
[2]TDS = total dissolved solids.
[3]NA = laboratory analysis cannot detect element concentration in water sample below a set range and all samples were below that set range.

Table 3.3. Ferrous iron (Fe^{2+}) concentrations in water treatment suspensions for drinking water and milk replacer suspension compared with formulated treatments

		Formulated Treatment (mg Fe/L)				
		0	**2**	**4**	**8**	**12**
Drinking water treatment	**Fe^{2+} Laboratory Analysis (mg/L)**	< 0.05	1.78	3.62	7.08	10.7
	± SD	NA	0.17	0.15	0.32	0.43
Milk replacer treatment water	**Fe^{2+} Laboratory Analysis (mg/L)**	< 0.05	1.80	3.62	7.04	10.2
	± SD	NA	0.13	0.27	0.53	0.81

Table 3.4. Effect of treatment on serum Fe concentration with an overall linear effect and a wk 2 linear effect

	Treatment, mg Fe/L						
	0	**2**	**4**	**8**	**12**	**SEM**	***P***
Serum Fe (µg/dL)	284.9	279.0	253.5	313.7	329.1	17.99	0.04
Week 2 serum Fe (µg/dL)	254.4	291.4	223.4	326.9	362.1	25.75	0.001
Week 4 serum Fe (µg/dL)	315.4	266.6	283.5	300.5	296.2	25.11	0.93

Table 3.5. Total daily dietary Fe consumption (mg/calf) from milk replacer suspension, starter pellet, and water treatment. Daily Fe from starter was back-transformed from square root and daily Fe from water treatment and total Fe consumption was back-transformed from natural log for presentation. Daily Fe consumption from starter different by wk ($P < 0.001$), from water treatment different by treatment ($P < 0.001$) and week ($P < 0.0001$), and total daily Fe consumption was significant by treatment ($P < 0.001$) and week ($P < 0.001$)

Dietary Fe Source	**Fe^{2+} Treatment (mg Fe/L)**					
Dietary Fe Source	**0**	**2**	**4**	**8**	**12**	**SEM**
Fe consumption from milk replacer suspension[1] (mg Fe/d)	137	151.2	165.4	193.8	222.2	NA
Fe consumption from starter[2] (mg Fe/d)	133.1	122.7	177.2	130.7	162.3	0.92
Fe consumption from water (mg Fe/d)	0	4.1	7.9	13.4	20.1	0.18
Total Fe consumption (mg Fe/d)	271.1	278.6	350.4	342.8	406.7	0.07

[1]Cow's Match WarmFront® BOV BM DBZ, Land O'Lakes Animal Milk Products Co., Shoreview, MN milk replacer accounted for 137 mg Fe/d for all treatments. Value calculated by adding average Fe concentration of the water used for milk replacer suspension to mg Fe in milk replacer powder.

[2]Ampli-Calf, Purina Animal Nutrition, St. Louis, MO formulated with 198 mg Fe/kg DM and provided *ad libitum*.

Table 3.6. Total intake of Fe (mg/calf per d) by week and treatment[1]

	Treatment, mg Fe/L					
Week		**0**	**2**	**4**	**8**	**12**
	0[2]	143	143	143	143	143
	1	193	209	247	261	327
	2	249	247	319	306	371
	3	288	322	386	370	421
	4	320	356	447	427	498

[1]Total mg Fe/d is sum of DMI of starter (198 mg Fe/kg), DMI of milk replacer powder (137 mg Fe/kg), drinking water intake by treatment, and milk replacer suspension water by treatment.

[2]Week 0 water calculated from average drinking water intake, water used for milk replacer suspension, and well water analysis of mg Fe/L (0.68 mg Fe/L) summed with starter DMI and milk replacer powder (122 mg Fe/kg).

Table 3.7. Ferrous iron (Fe^{2+}) intake from water treatments as a percentage of recommended Fe intake[1] (mg Fe/calf per d) by wk and treatment

	Treatment, mg Fe/L					
		0	**2**	**4**	**8**	**12**
Week	**0**	-44[2]	-44	-44	-44	-44
	1	-7	10	48	61	127
	2	15	14	86	73	138
	3	16	50	114	98	149
	4	14	50	141	121	193

[1]Recommended mg Fe/d calculated by NRC (2001) recommendation of 150 mg Fe/kg DM. The DM included starter (198 mg Fe/kg) and milk replacer powder (122 mg Fe/kg) and was calculated using DMI by wk and treatment summed with mg Fe/d from drinking water and milk replacer suspension treatment.
[2]Negative value indicates mg Fe/d below that calculated by NRC (2001).

Table 3.8. Ferrous iron (Fe^{2+}) as a percentage of total Fe consumed

	Treatment, mg Fe/L					
		0	**2**	**4**	**8**	**12**
Week	**0**	76	76	74	74	74
	1	96	105	124	131	164
	2	107	106	137	131	159
	3	106	118	142	136	155
	4	105	116	146	140	163

Assumes that Fe from starter (198 mg Fe/kg) and milk replacer powder (122 mg Fe/kg) is of Fe^{3+} valence and that of free drinking water treatment and milk replacer suspension water treatment is of Fe^{2+} valence.

Table 3.9. Trend for effect of treatment on average daily gain (ADG = kg/calf per d; $P = 0.08$)

	Treatment, Mg Fe/L					
	0	**2**	**4**	**8**	**12**	**SEM**
ADG	1.05	0.97	1.21	1.07	1.09	0.06

Table 3.10. Body measurements of calves on each Fe^{2+} treatment at start of experiment (0), and 2 and 4 wk of experiment

	Week			
	0	**2**	**4**	**SEM**
Weight (kg)[1]				
Bull	64.9	77.4	94.5	1.0
Heifer	61.2	76.8	91.0	0.8
Hip Height (cm)[2]				
Bull	82.5	87.7	90.4	2.1
Heifer	83.2	86.6	91.2	1.8
Hip Width (cm)				
Bull	25.0	24.0	24.7	0.7
Heifer	22.9	24.6	25.2	0.6
Wither Height (cm)[3]				
Bull	80.1	84.2	86.5	1.4
Heifer	79.7	83.3	88.0	1.5
Heart Girth (cm)[4]				
Bull	95.2	101.7	108.0	0.5
Heifer	93.0	99.6	106.9	0.4
Body Length (cm)[5]				
Bull	89.0	91.1	98.4	0.7
Heifer	85.6	91.1	98.2	0.8

[1]Effect of wk on BW ($P < 0.001$).
[2]Effect of wk on hip height ($P = 0.04$).
[3]Effect of wk on wither height ($P = 0.04$).
[4]Effect of wk ($P < 0.0001$) and sex ($P = 0.01$) on heart girth.
[5]Effect of wk on body length ($P < 0.001$).

FIGURES

Figure 3.1. Treatment by wk interaction on serum Fe concentration (P = 0.05, SEM = 25.4) with wk 0 data used as a covariate

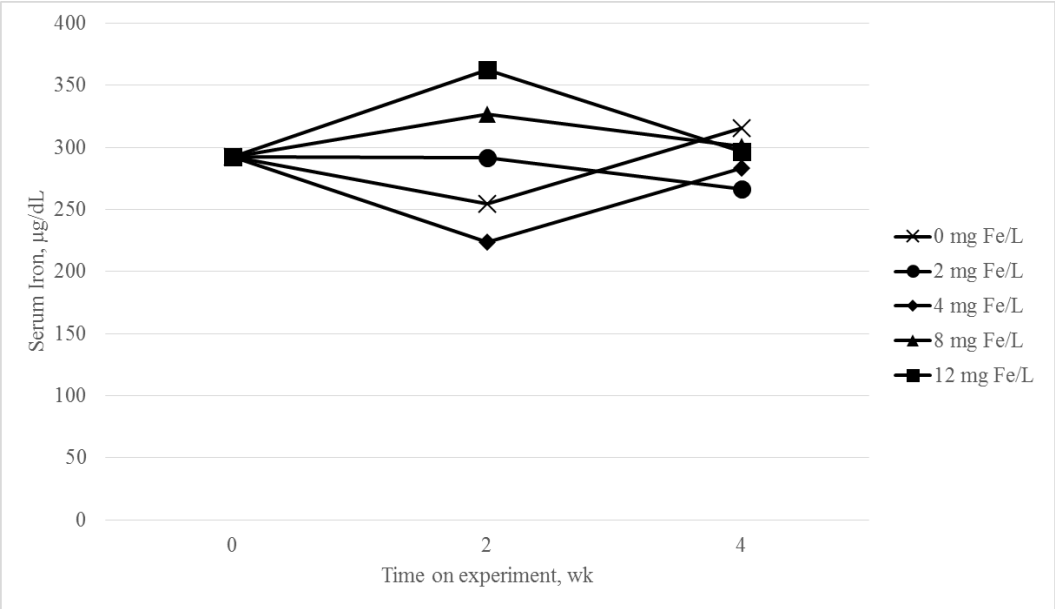


Figure 3.2. Total iron-binding saturation percentage for each ferrous iron treatment (P = 0.02, SEM = 2.66) with an overall linear effect (P = 0.01) and a wk 2 linear effect (P < 0.001)

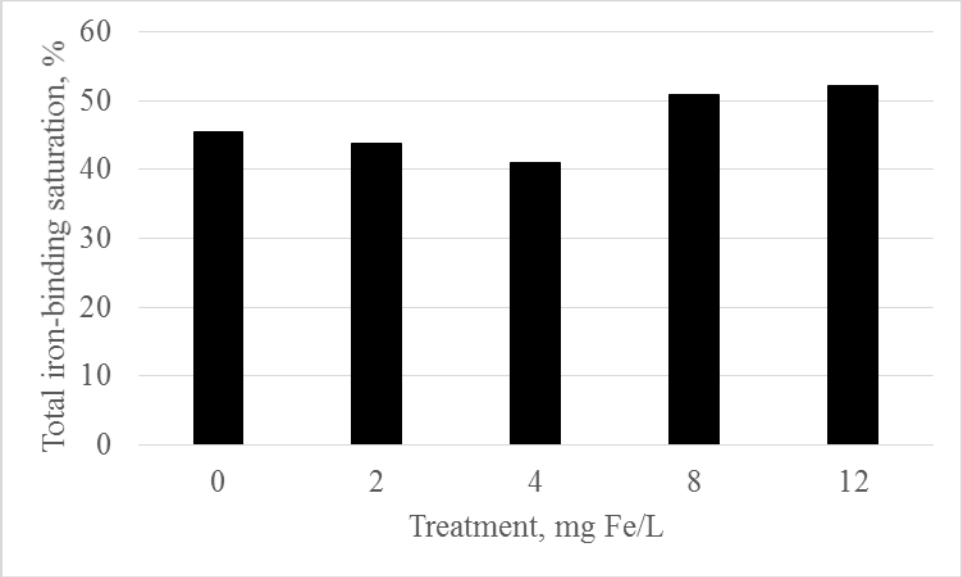


Figure 3.3. Treatment by wk interaction on total iron-binding saturation percentage (TIBS; P = 0.03, SEM = 3.65) with wk 0 data as covariate

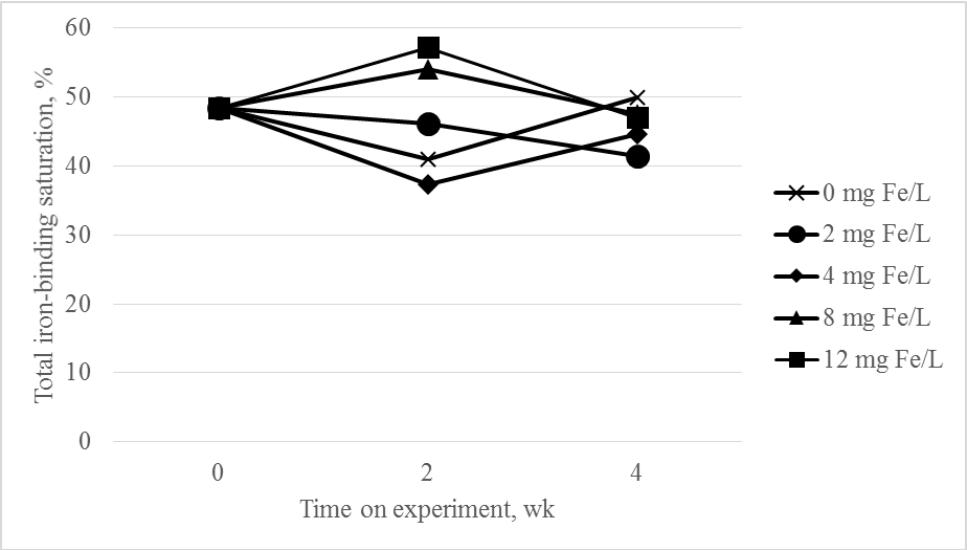


Figure 3.4. Effect of treatment on hemoglobin concentration (P = 0.01) with a quadratic response over time (P = 0.003)

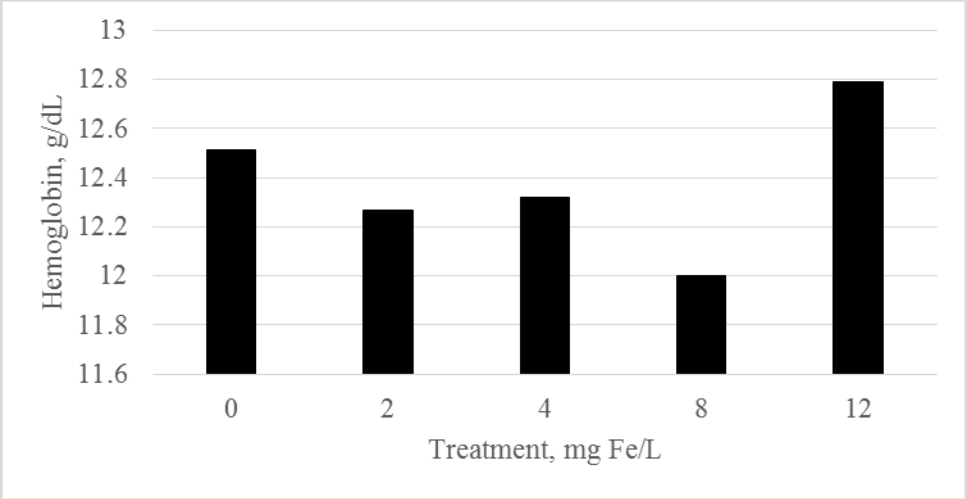


Figure 3.5. Water intake of pre-weaned calves by week of experiment (P < 0.001, SEM = 0.07) with d 25-27 of the standardization period as covariate. Water intake transformed to natural log for statistical analysis and then back-transformed for presentation in the figure. Week 1 = 28 to 34 d of age, week 2 = 35 to 41 d of age, week 3 = 42 to 48 d of age, week 4 = 50 to 56 d of age

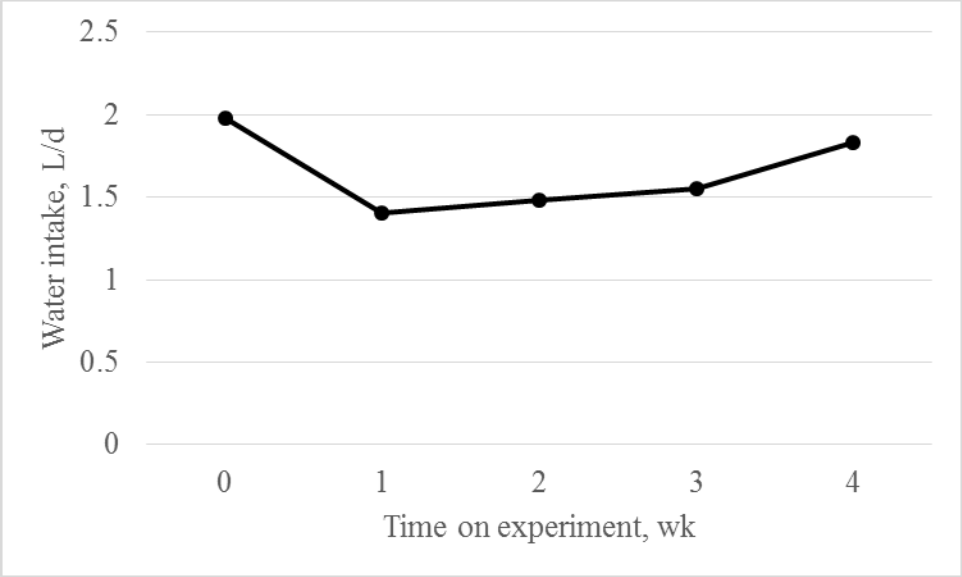


Figure 3.6. Starter intake of calves by week of experiment (*P* < 0.001, SEM = 0.04) with d 25-27 of the standardization period as covariate. Starter intake transformed to square root for statistical analysis and then back-transformed for presentation in the figure. Week 1 = 28 to 34 d of age, week 2 = 35 to 41 d of age, week 3 = 42 to 48 d of age, week 4 = 50 to 56 d of age

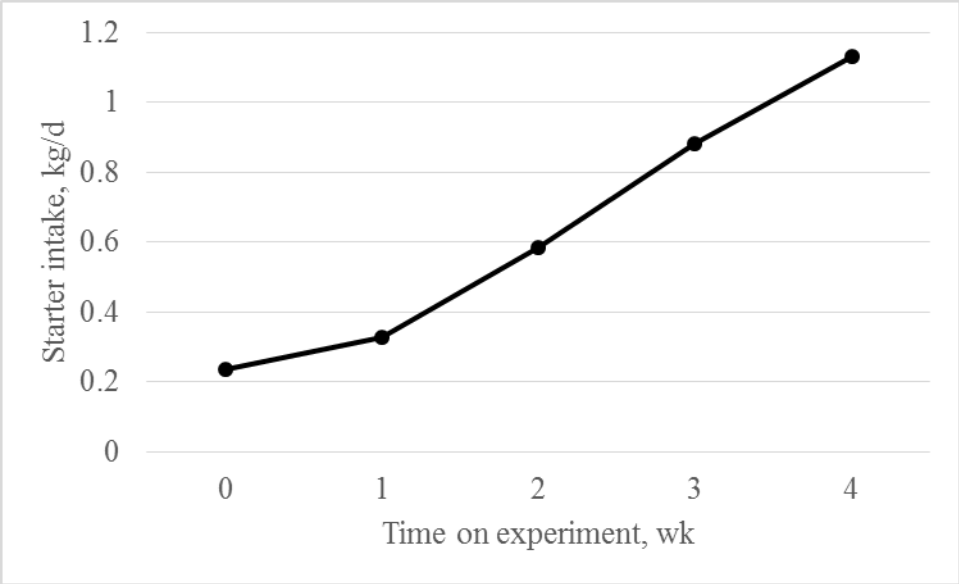


Figure 3.7. Effect of treatment on body weight (P = 0.01; SEM = 0.99)

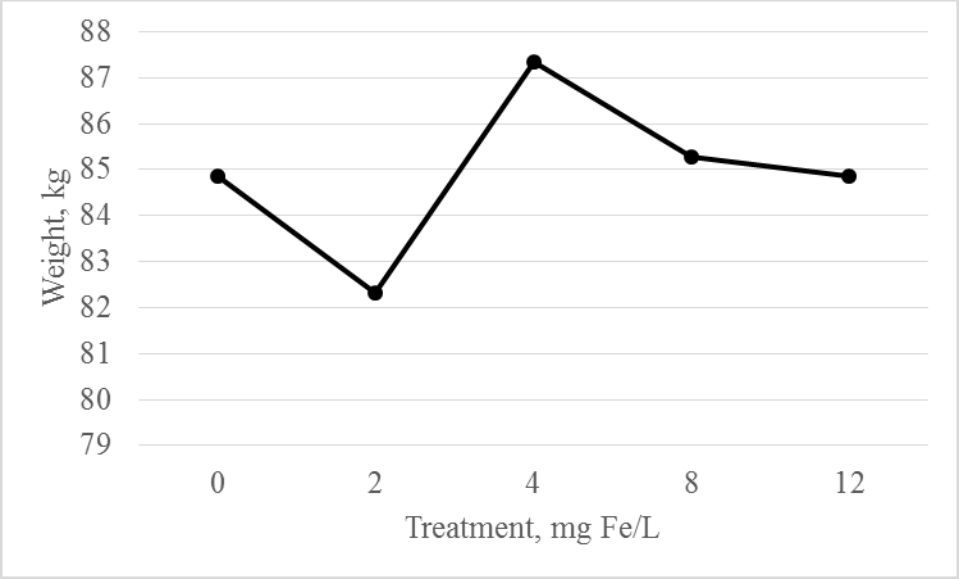


Figure 3.8. Trend for a sex by week of experiment interaction on calf body weight (P = 0.07, SEM = 1.05) with wk 0 data used as a covariate

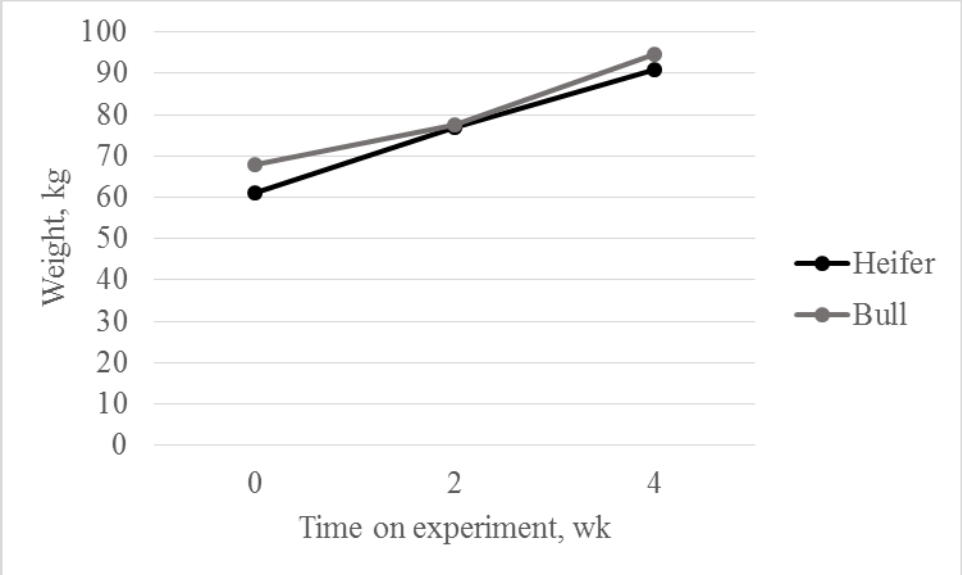


Figure 3.9. Effect of treatment on serum calcium (P = 0.001, SEM = 0.13) with a quadratic effect of treatment (P = 0.001)

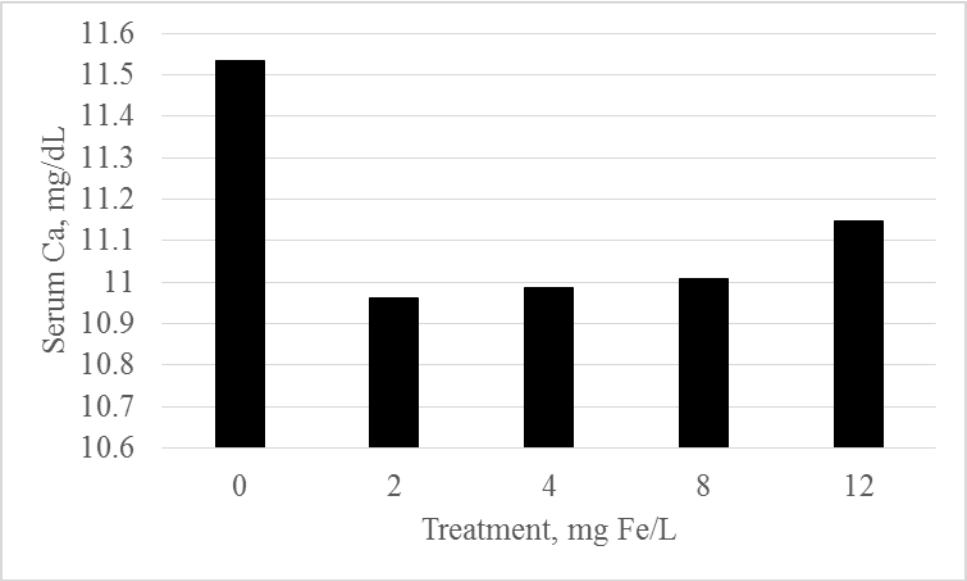


Figure 3.10. Effect of treatment on serum calcium with a wk 2 quadratic effect of treatment (P = 0.02) and a wk 4 quadratic effect of treatment (P = 0.02)

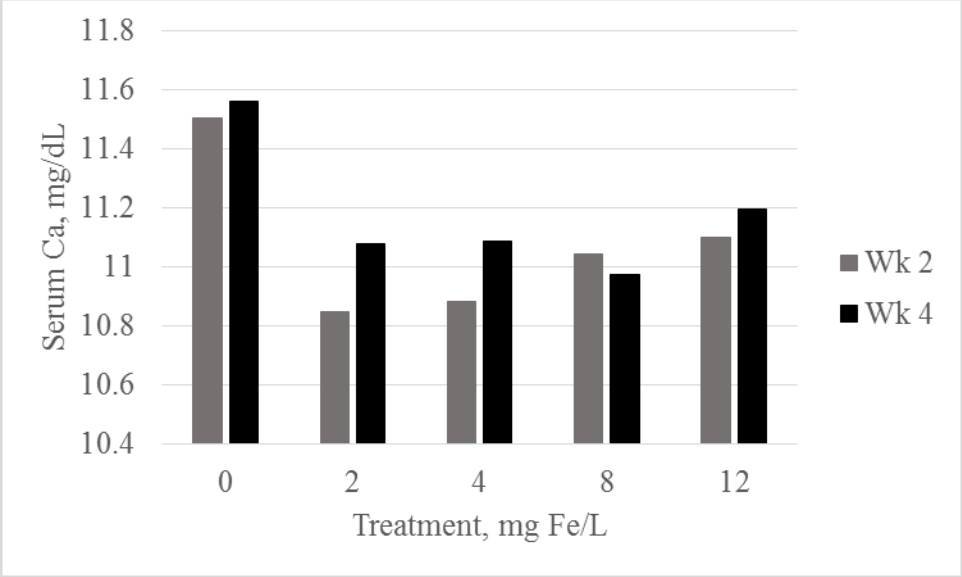


Figure 3.11. Trend for treatment by sex interaction on serum P (*P* = 0.05, SEM = 0.21)

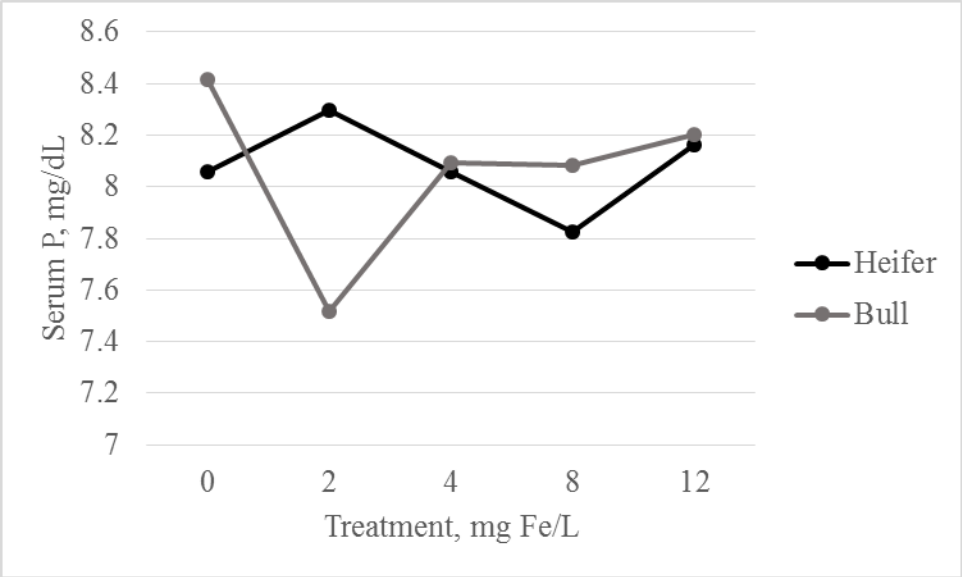


Figure 3.12. Trend for week by sex interaction on **s**erum phosphorus (*P* = 0.08, SEM = 0.14) with wk 0 data as covariate

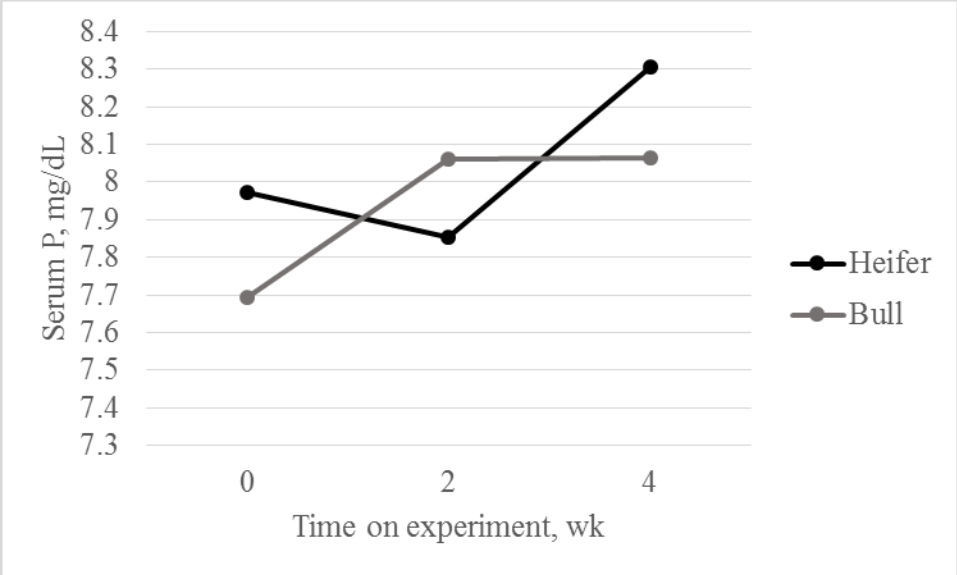


Figure 3.13. Effect of treatment on hematocrit percentage ($P = 0.005$) with a quadratic effect of treatment over time ($P < 0.001$)

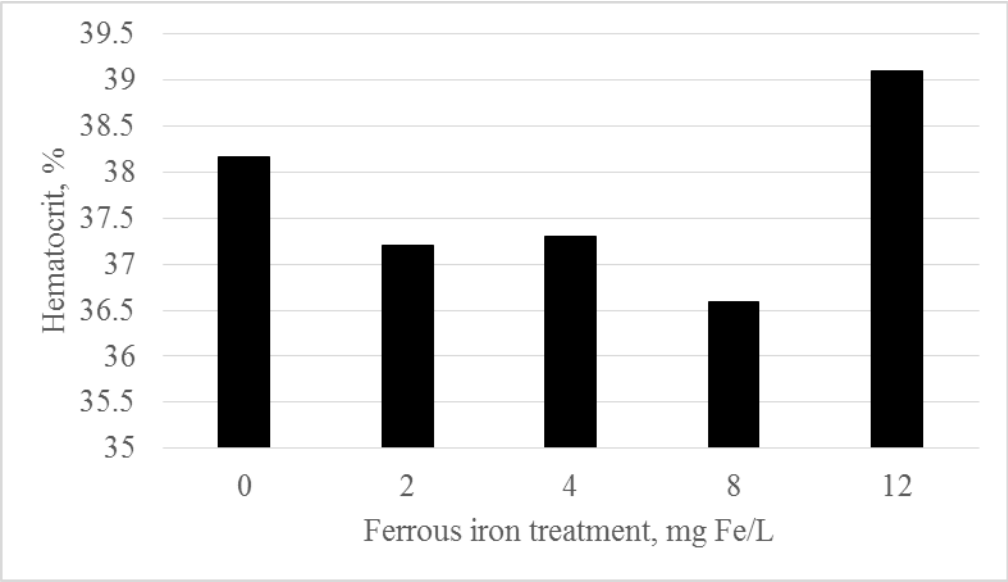


Figure 3.14. Quadratic effect of treatment of treatment at wk 4 ($P = 0.002$) on hematocrit with a trend for a quadratic effect of treatment of treatment at wk 2 ($P = 0.06$)

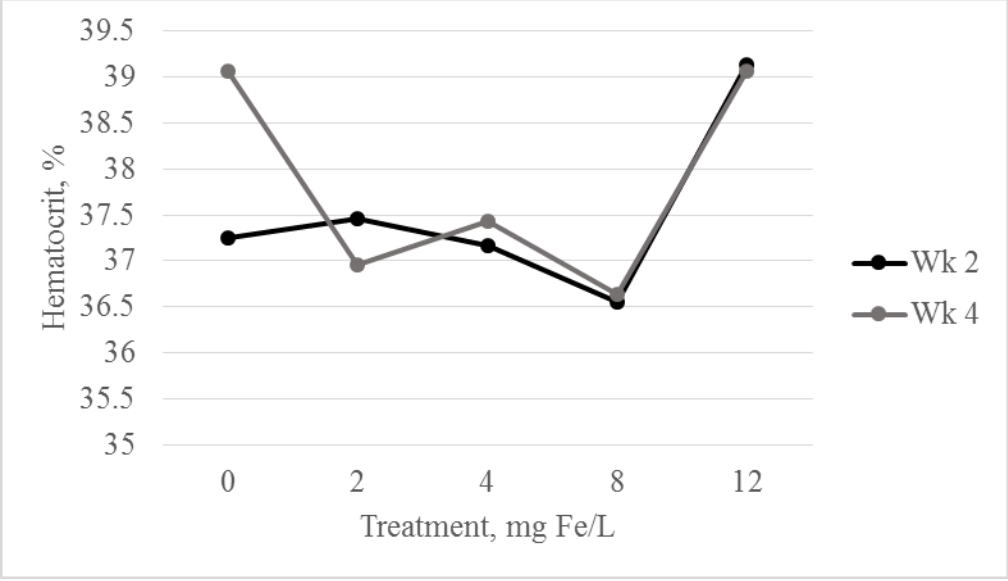


Figure 3.15. Effect of treatment on mean corpuscular hemoglobin (P = 0.05, SEM = 0.11) with an overall trend for a cubic effect of treatment (P = 0.01)

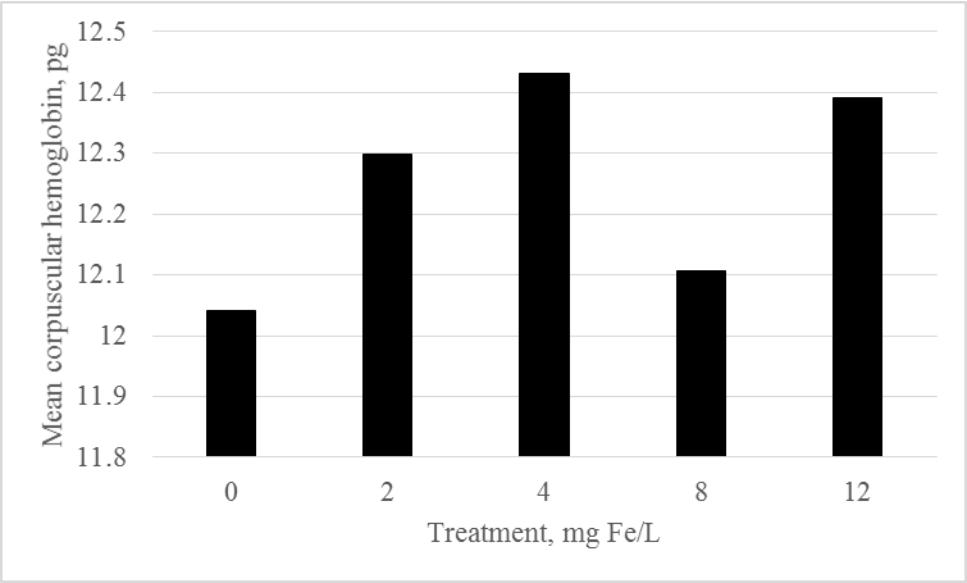


Figure 3.16. Effect of treatment on mean corpuscular hemoglobin (P = 0.05, SEM = 0.11) with a trend for a cubic effect of treatment at wk 2 (P = 0.06) and a cubic effect of treatment at wk 4 (P = 0.04)

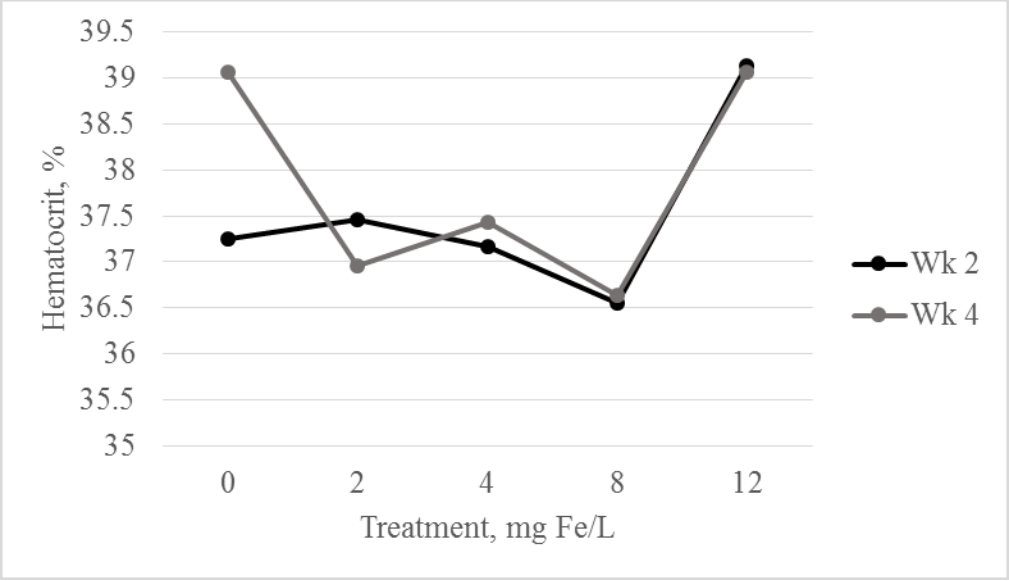


Figure 3.17. Effect of treatment on red blood cell distribution width percentage (P = 0.04, SEM = 0.36) with a quadratic effect of treatment of treatment overall (P = 0.01)

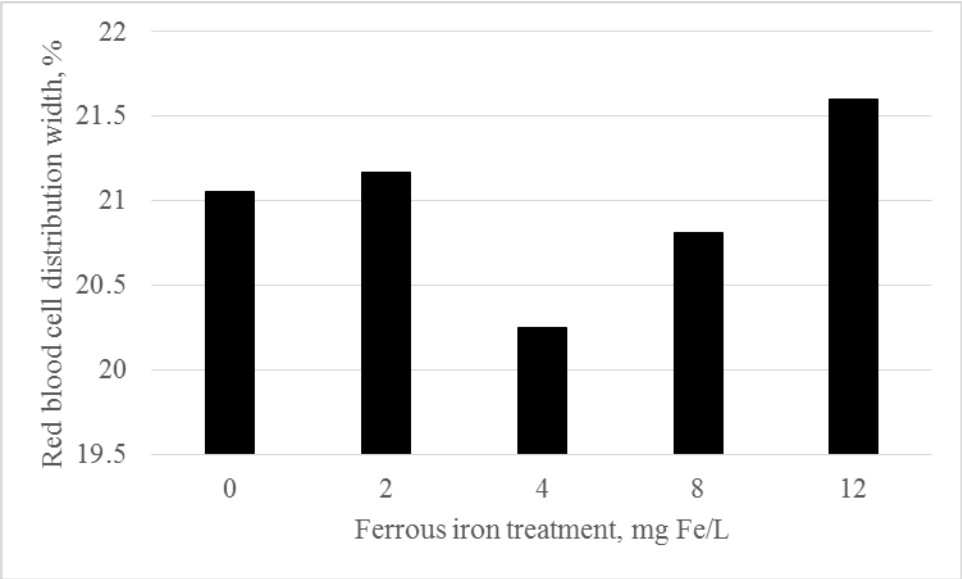


Figure 3.18. Effect of treatment on red blood cell distribution width percentage (P = 0.04, SEM = 0.36) with a trend for a quadratic effect of treatment at wk 2 (P = 0.09) and wk 4 (P = 0.07)

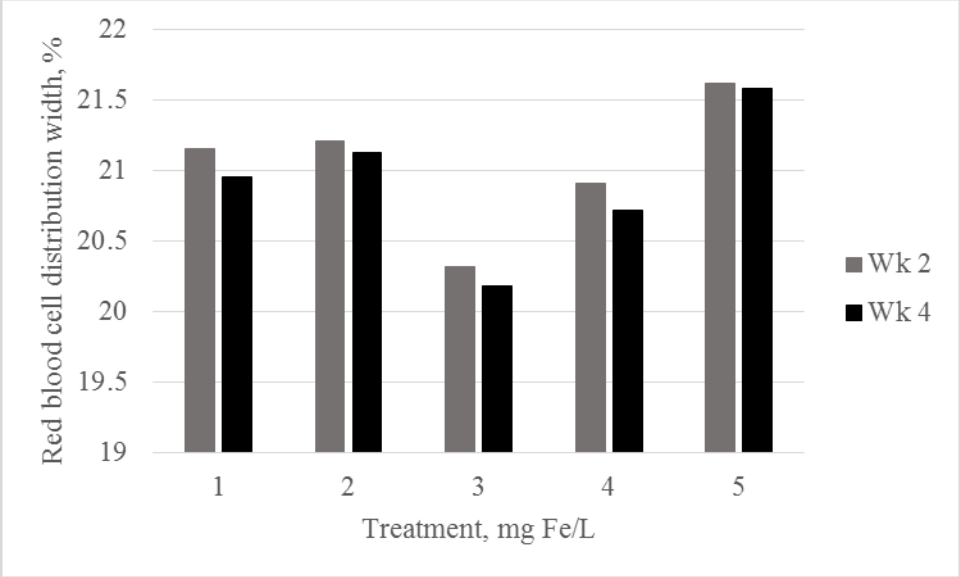


Figure 3.19. Effect of treatment on platelet count (P = 0.01, SEM = 17.7) with an overall linear effect of treatment (P = 0.03)

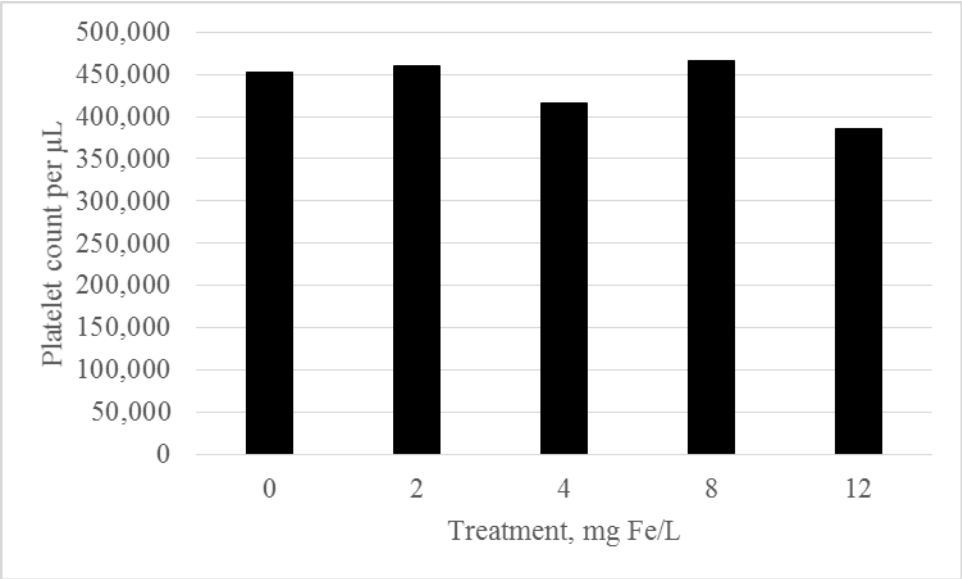


Figure 3.20 Effect of treatment on platelet count (P = 0.01, SEM = 17.7) with a trend for a wk 2 linear effect (P = 0.08)

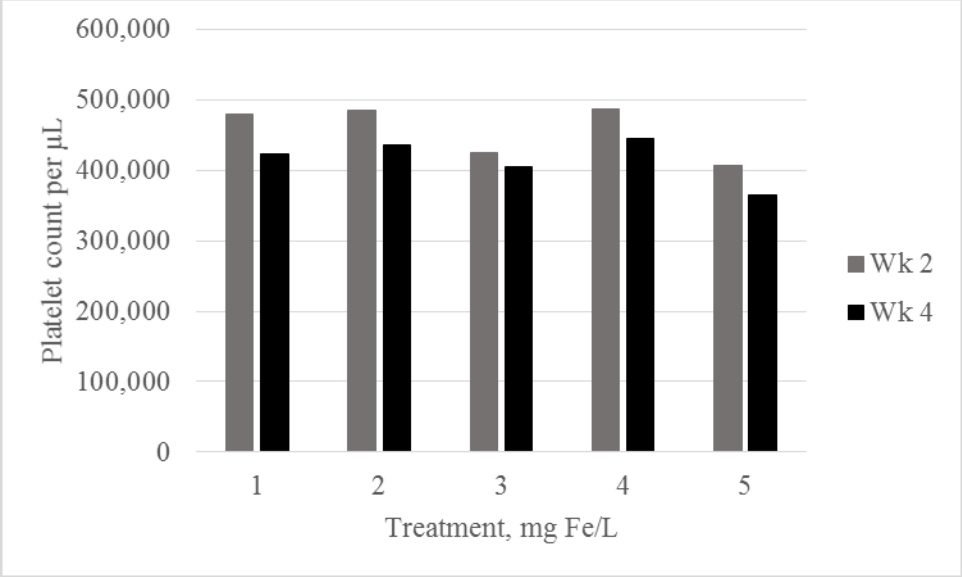


Figure 3.21. Effect of Fe treatment on monocyte count (P = 0.03, SEM = 0.05) with an overall quadratic effect of treatment (P = 0.002)

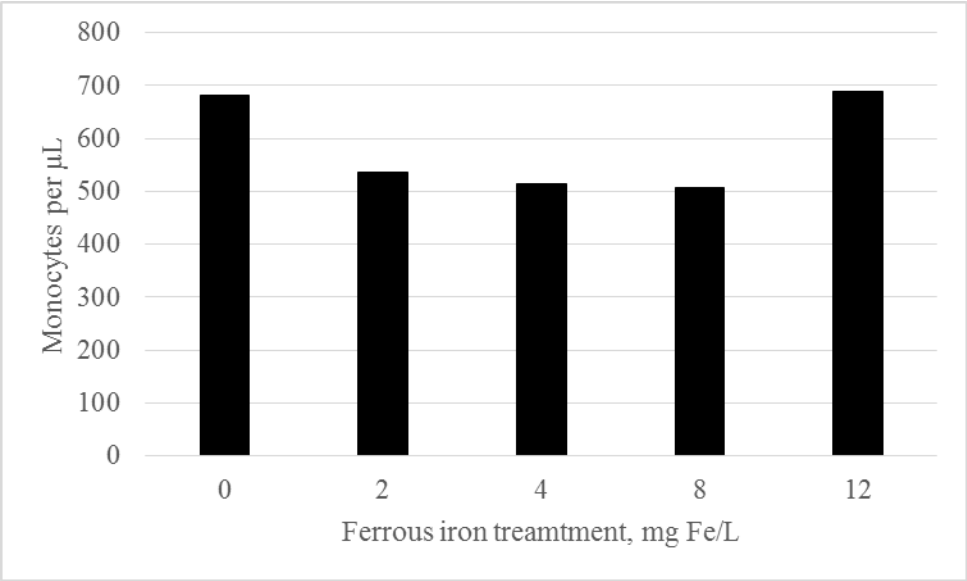
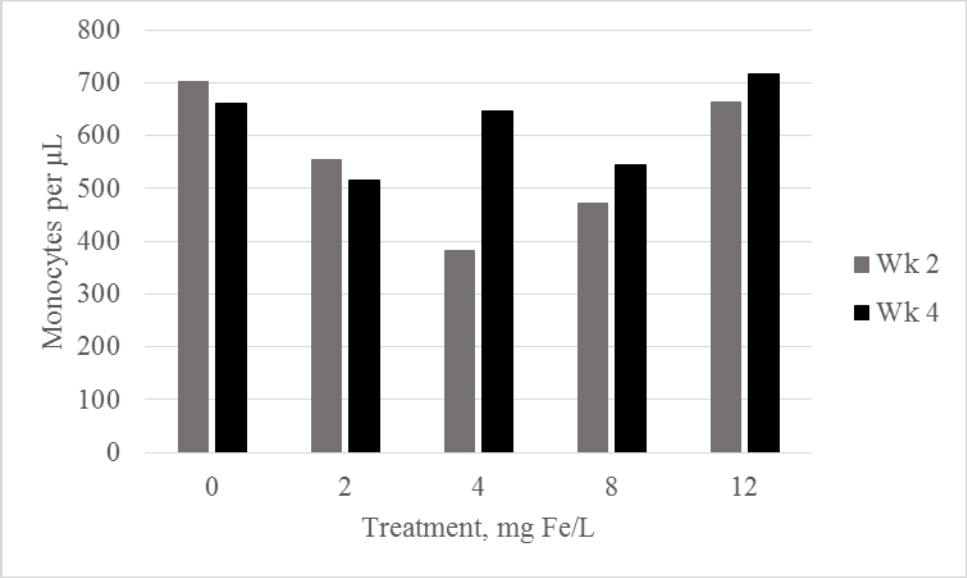


Figure 3.22. Effect of Fe treatment on monocyte count (P = 0.03, SEM = 0.05) with a quadratic effect of treatment at wk 2 (P = 0.002)



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CHAPTER 4

CONCLUSIONS AND IMPLICATIONS

Ground water contains Fe that is primarily in the ferrous valence (Fe^{2+}). This form of Fe is more biologically available than the ferric form (Fe^{3+}) and can be more readily absorbed by calves. Typical dairy calf milk replacer and starter pellet feeds are formulated to meet or exceed the Fe requirements of a growing calf. Any Fe^{2+} consumed from ground water via milk replacer suspension and free drinking water would be considered in excess of a calf's needs. Anecdotal information suggests this excess consumption of Fe^{2+} might negatively impact calf water intake and growth. We hypothesized that greater Fe^{2+} concentrations in water could affect calves through decreased water intake and showing preference to water without Fe^{2+}, and through changes in serum Fe status, dry matter intake, and growth. Our objectives were to test preferences of pre- and post-weaned calves in two experiments, and in a third experiment assess serum Fe status, starter and water intake, and growth of calves.

In Chapter 2, we presented results from two different experiments that evaluated pre- and post-weaned calves' ability to rank six concentrations of Fe^{2+} water (0, 2, 4, 8, 12, and 20 mg Fe/L) in a non-parametric sequential elimination ranking design. There was below "substantial agreement" among calves in both experiments. All post-weaned calves in experiment 2 ranked 0 mg Fe/L water 1st (preferred to drink it most and first most often), and showed fair agreement in ranking the 12 mg Fe/L treatment last. These experiments suggest that pre-weaned calves do not have predilection towards water with or without Fe^{2+}, though post-weaned calves do prefer water with lower Fe^{2+} concentrations when made to choose against water with a greater Fe^{2+} concentration.

Overall, results from the first two experiments suggest that calves do have the ability to choose among waters with different concentrations of Fe^{2+}. However, total water consumption did not appear to be detrimentally affected by concentrations of Fe^{2+} up to 20 mg Fe/L. We suspect that pre-weaned calves in experiment 1 did not consume enough water to substantially rank preferences for the water treatments. Repeating the experiment with the same calves once they are weaned would be of interest to determine if a more substantial preference of water develops as they naturally consume more water. Future research should investigate calves' ability to rank water with Fe^{2+} at multiple life stages using the same calves to determine how preferences might develop or change as water consumption increases with age. Also, possibly in the future studies testing greater concentrations of Fe^{2+} in water might be of value.

In Chapter 3, we presented results from an experiment designed to investigate the effects of increased Fe^{2+} in drinking water and milk replacer suspension on serum Fe status, dry matter intake, water intake, and growth of pre-weaned calves. This was to assess if increased Fe^{2+} in the water would cause a decrease in water intake leading to decreased starter intake and poor growth. Starter pellet intake and water intake increased over time as calves grew. Calves provided the 4 mg Fe/L treatment had greater BW than calves on other treatments; however, hip height, wither height, heart girth, and hip width were not affected by treatment. These results suggest that increased Fe^{2+} consumption from drinking water and milk replacer suspension can affect Fe status of calves between 28 and 56 d of age.

Overall, there were no detrimental effects of Fe^{2+} on calves over the course of the experiment. Serum Fe, TIBS, and measures of hemoglobin and hematocrit (MCH, MCV, and MCHC) stayed within ranges considered normal for calves of this age. Future research should evaluate the effects of greater Fe^{2+} concentrations in water as the calf ages. Many of the hematological variables measured in this experiment are in various states of physiological

change as the calf ages and many of these variables have been reported to have great variation up to 3 mo of age. It would be of interest to monitor the Fe status of calves at this time once their biological systems have narrowed towards adult reference standards to see if Fe^{2+} consumed from water has more of a detrimental effect on their health.

The current upper tolerable recommendation of 0.3 mg Fe/L for dairy cows (NRC, 2001) was borrowed from the EPA (2004) water quality standard for water aesthetically pleasing for human consumption. Through a series of experiments, our research indicates that pre-weaned calves do not substantially show preference for Fe-free water, and that Fe status of pre-weaned does not appear to be detrimentally affected at concentrations up to 12 mg Fe/L. However, post-weaned calves that consumed greater amounts of water do show preference for Fe-free water and it would be of interest to evaluate whether or not Fe status continues to change when provided Fe^{2+} water after 56 d of age.

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REFERENCES

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